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VOLUME 47

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ENDOCRINOLOGY

VOLUME 47 JULY, 1950 NUMBER 1

AN ANTIDIURETIC SUBSTANCE IN THE BLOOD OF NORMAL AND ADRENALECTOMIZED RATS[1,2]

JAMES H. BIRNIE, W. J. EVERSOLE, W. R. BOSS, C. M. OSBORN AND ROBERT GAUNT

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PRELIMINARY reports from this laboratory have described a procedure which demonstrates the presence of a labile antidiuretic, chloruretic substance(s) in the blood serum of normal and adrenalectomized rats (Birnie *et al.*, 1949a; 1949b; Birnie and Boss, 1949). The purpose of this report is to describe the biological properties of the antidiuretic substance (ADS) found in rat serum and to compare its actions with those of the posterior pituitary antidiuretic hormone. While the pituitary origin of serum ADS is not considered to be conclusively established by the evidence presented below, the results are most easily interpreted on the basis of such an hypothesis.

It is important to keep in mind that all of the tests reported here are on rat serum tested in rats. We have not as yet made extensive or systematic attempts to test serum from one species in another.

THE TESTING PROCEDURE AND OTHER METHODS

To test samples of blood serum for their antidiuretic content, male rats weighing approximately 200 gms. were fasted but allowed water for 18 hours prior to hydration. Two doses of water, each consisting of 3 ml. per 100 sq. cm. of body surface, warmed to body temperature, were then administered at hourly intervals to such animals in individual metabolism cages. For a 200 gm. rat each dose was 9 ml. as calculated according to the formula of Benedict (1938). One hour after the administration of the second dose of water the urine was measured. Any rat which, during the two hour hydration period,

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[1] This investigation was supported by a research grant from the National Heart Institute, U. S. Public Health Service.

[2] We are indebted to Mr. F. E. Houghton, Ciba Pharmaceutical Products, Inc., for DCA; and to Dr. Dwight J. Ingle, the Upjohn Company, for the adrenal cortical extract and the gelatin solution used in these experiments.

had a urine excretion rate 50 per cent more than or less than the mean of the groups of 6 was discarded. The rats with a normal diuretic response were then placed over clean funnels, the test material injected intraperitoneally, and a third dose of water administered. Following the third dose of water the urine output was measured at 30 minute intervals for a period of 3 hours. The water excreted at any period was calculated as the per cent of the total water given (3 doses) minus that excreted prior to injection of the test material. The percentage of water excreted at 90 minutes after the injection of the test material was adopted as the definitive figure, and in ordinary experiments there is actually little gained by continuing observations beyond that time.

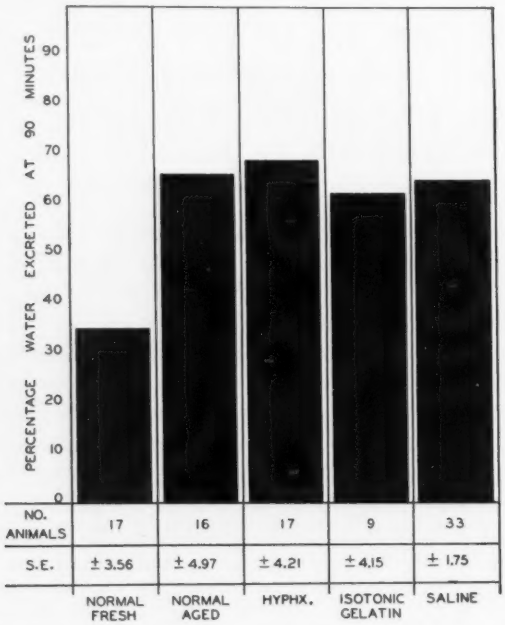

FIG. 1. Showing the effect on water excretion produced after 90 minutes by the injection of hydrated test animals with 1 ml. samples of serum from normal and hypophysectomized rats.

It was found that this method, designed not so much to equalize hydration as to establish a high diuretic rate, permitted better determinations of ADS than the ordinary Burn assay procedure. It is our impression that the reliability of the testing method was considerably enhanced if: (1) rats were used that were well adapted to laboratory conditions, (2) if the stomach-tubing was done by an experienced person to insure speed and minimal disturbance of the animal, and, (3) if the animals were kept in a quiet place during the test. No rat was used for more than one test although the necessity of this precaution was not demonstrated.

To obtain serum for the test, blood was taken directly from the heart without the use of an anticoagulant after the induction of ether anesthesia. The blood was briefly cen-

trifuged (about 2 minutes), serum decanted, and immediately injected into test animals unless otherwise specified. By this method the serum was injected within 5 minutes after it was drawn. Speed is essential because of the labile nature of the ADS, and we have gained the impression, although the point was not systematically studied, that activity is lost more rapidly when serum remained in contact with the clot than otherwise.

Urine chlorides were determined by the method of Van Slyke and Snedroy (Peters and Van Slyke, 1932). Unless otherwise specified, determinations were made on the urine collected over the entire 3 hour test period.

Creatinine clearance determinations were used as a measure of the effect of serum samples on glomerular filtration, the technique used being essentially that of Lotspeich (1949), modified only in the volume of water administered. The clearance period was of 30 minutes duration immediately following the injection of test serum.

RESULTS

Antidiuretic, chloruretic activity. The injection of 1 ml. of fresh serum from normal animals depressed the rate of urine flow and increased chloride excretion (Figure 1 and Table 1). If the decanted serum samples were allowed to stand at 5 to 90°C. for 12 hours the antidiuretic and chloruretic activity was lost (Figure 1 and Table 1). While detailed studies are lacking it has become apparent from incidental observations that the

TABLE 1. SHOWING MEAN EFFECT ON CHLORIDE (AS NaCl) EXCRETION PRODUCED BY VARIOUS TYPES OF BLOOD SERUM

Types of serum tested (1 ml. sample)	Number test animals	Chloride excreted	
		mg./ml. ±S.E.	Total in 3 hours, mg. ±S.E.
Normal fresh.	17	1.06±0.12	11.1±1.19
Adrenalectomized, 3–7 days, fresh.	26	1.14±0.09	12.3±1.10
Adrenalectomized, 9–12 days, fresh.	10	1.29±0.40	13.2±1.59
Normal drawn 12 hours.	16	0.45±0.04	5.6±0.76
Adrenalectomized drawn 12 hours.	5	0.55	7.1
Hypophysectomized, 6–11 days, fresh.	11	0.62±0.95	8.5±1.42
Normal Saline (no serum).	35	0.49±0.036	7.2±0.62
Egg Albumin 5%	8	0.34	3.0
Isotonic gelatin.	8	0.34	4.8

antidiuretic properties of rat serum are lost rapidly at room temperature; appreciable reductions in activity have been noted within 30 minutes (Table 2).

Effect of anesthesia. To test the possibility that anesthesia might alter the serum ADS level, blood samples obtained without anesthesia or under Nembutal were compared with those drawn from etherized animals. It was found that the serum ADS level was essentially the same regardless of whether or not the donor rat was under anesthesia. In 10 cases the mean percentage water excreted at 90 minutes by rats injected with serum from nonanesthetized animals was 42.5±4.8; when serum obtained under Nembutal anesthesia was used in 10 cases the percentage excretion was

38.3 ± 4.2; 7 animals receiving serum from etherized donors and run at the same time excreted 41.1 ± 3.1 per cent. Saline-treated controls for this series (8 cases) excreted 71.4 ± 5.6 per cent. As the collection of blood is easier when the rat is under an anesthetic this method was used routinely in obtaining samples.

Renal clearance tests. Following the injection of 1 ml. of fresh serum from normal rats into hydrated test animals there was a slight but significant increase in glomerular filtration within 30 minutes after administration as indicated by creatinine clearance studies. During the same period

TABLE 2. SHOWING MEAN EFFECT ON GLOMERULAR FILTRATION AND URINE FLOW PRODUCED BY VARIOUS TYPES OF BLOOD SERUM

Type of serum tested (1 ml. sample)	Number test animals	Creatinine clearance ml./100 gms./min.	Urine flow ml./100 gms./min.
Fresh serum.	26	1.23 ± 0.036	0.026
Serum drawn 30 min.	16	1.09	0.046
Aged serum (12 hrs.).	8	1.10	0.052
Controls (no serum).	20	1.00 ± 0.036	0.059

there was a marked decrease in the rate of urine flow (Table 2). These results indicate that the action of serum is exerted by increasing the tubular reabsorption of water rather than by decreasing glomerular filtration. When the serum was allowed to stand for 30 minutes at 23°C. before being injected into test animals it caused no alteration in glomerular filtration and had lost a considerable portion of its antidiuretic activity (Table 2).

Serum activity after hypophysectomy. No antidiuretic action was observed when 1 ml. samples of serum from rats which had been hypophysectomized for 6 to 11 days were injected into hydrated test animals. Results with serum from hypophysectomized animals were essentially the same as when normal saline solution was injected instead of serum (Figure 1 and Table 1). This observation indicates that the antidiuretic material present in the serum of intact animals is of pituitary origin or requires the presence of the pituitary for its expression.

Chloride concentration in the urine of animals receiving serum from hypophysectomized rats was significantly lower than in cases receiving serum from normal animals. The differences in total chloride were not clearly significant due perhaps to the inherently high variability of chloride excretion in such experiments.

Inactivation by thioglycollic acid. Thioglycollic acid was neutralized to pH 7.5 with NaOH and mixed with an equal volume of test material 5 minutes before being injected into hydrated rats. Neutralized thioglycollic acid caused the inactivation of antidiuretic substance in fresh serum (Table 3) in the same manner that it did equivalent amounts of posterior

TABLE 3. SHOWING THE INACTIVATION OF SERUM AND PITRESSIN BY THIOGLYCOLLATE

Type of test material	Number test animals	Percentage water excreted at 90 min.
Fresh serum + water.	5	32.3
Fresh serum + thioglycollic acid.	7	53.1
Saline + thioglycollic acid.	3	53.7
Pitressin (5 milliunits) + thioglycollic acid.	3	49.5
Pitressin (5 milliunits) + water	3	16.3

pituitary extract (Pitressin). While this observation does not prove that the serum ADS and Pitressin are identical substances it suggests a chemical similarity between the two. This experiment was kindly suggested by Dr. H. B. van Dyke and is based upon his previous work (van Dyke *et al.*, 1942).

Subcutaneous administration of serum. When serum was injected subcutaneously its effect was less pronounced than when it was given by the usual intraperitoneal route. The effectiveness of Pitressin was likewise reduced when it was given subcutaneously (Table 4).

TABLE 4. SHOWING THE MEAN EFFECT OF SUBCUTANEOUS AND INTRAPERITONEAL INJECTION OF FRESH SERUM AND PITRESSIN

Route of administration	Number test animals	Percentage water excreted at 90 min.
Fresh serum, intraperitoneal.	9	37.6
Fresh serum, subcutaneous.	16	47.1
Pitressin (5 mu), intraperitoneal.	8	16.7
Pitressin (5 mu), subcutaneous.	11	44.2

Some control experiments. Two types of foreign protein were injected into hydrated test animals as a control measure to see if the effects of rat serum were specific. In the first case, 1 ml. samples of isotonic gelatin solution (Upjohn) were injected intraperitoneally and had no effect on the rate of diuresis or on chloride excretion (Figure 1 and Table 1). This was a bland solution and caused no obvious discomfort to the animal. In the other case, 1 ml. of a 5 per cent solution of crude, commercial egg albumin was given. This material inhibited diuresis, but differed from serum in that it failed to cause chloruresis (Table 1) and its antidiuretic action was not exhibited until about 30 minutes after injection. Unlike gelatin or serum, it caused severe distress; the animals were obviously very uncomfortable after its injection and the antidiuresis seen was probably due to circulatory disturbance. The experiment provided a clean-cut example of the distinction between the action of serum and a superficially similar action of nonspecific irritating materials.

Actually what was probably the nearest ideal control solution used in

these experiments was blood serum inactivated by aging overnight under refrigeration. As noted above, it exerts none of the effects of fresh serum.

Serum activity after adrenalectomy. Serum from animals adrenalectomized for 3 to 7 days contained more antidiuretic activity than that of normal animals (Figure 2). Chloruretic activity may have been increased, although statistical proof was lacking (Table 1). The figures suggest, but do not

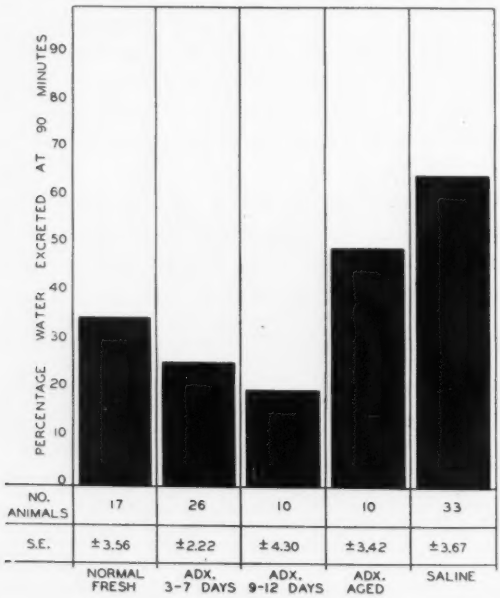

FIG. 2. Showing the effect on water excretion produced after 90 minutes by the injection of hydrated test animals with 1 ml. samples of serum from adrenalectomized rats.

prove, that the antidiuretic activity of serum is still further enhanced at 9 to 12 days after adrenalectomy (Figure 2).

If the decanted serum samples from adrenalectomized animals were allowed to stand at 5 to 9°C. for 12 hours a portion of the antidiuretic activity was lost. Such serum, however, did not loose its activity completely as does the serum from normal animals (compare Figure 1 and Figure 2). One possible interpretation of this result is that in adrenalectomized animals there is a deficiency in the system which normally serves to inactivate circulating antidiuretic substance (see Discussion).

The accumulation of ADS after adrenalectomy can be prevented by the administration of cortical extract (2 ml. per day in drinking water), the injection of 0.5 mg. DCA daily, or the use of 1 per cent saline as drinking

fluid (Table 5). It is of interest to note that while all of these procedures will return the antidiuretic content of adrenalectomized animals' blood essentially to normal levels they do not reestablish the normal rate of water excretion (Gaunt, 1946). The probable basis of this fact is discussed in a subsequent section.

TABLE 5. SHOWING THE EFFECT OF SALINE AND ADRENAL STEROID THERAPY ON SERUM ADS LEVEL OF RATS ADRENALECTOMIZED FOR 7 DAYS

Treatment of animals from which serum was drawn	Number test animals	Percentage water excreted at 90 min.
NaCl, 1% drinking fluid.	17	38.4
DCA, 0.5 mg. per day.	12	38.3
Adrenalectomized untreated.	26	26.0
Intact controls.	17	35.0

Serum activity of intact animals treated with saline or adrenal steroids. An attempt was made to determine whether the serum ADS level of intact rats could be lowered by the administration of such agents as saline, DCA, and cortical extract. None of the procedures tried did do so (Table 6) in spite of the fact that doses of DCA sufficient to produce adrenal atrophy were employed. In a few instances there were suggestions that treatment might increase the serum antidiuretic activity, but in view of the small number of cases involved this was not a certain observation. It is reminiscent of the fact, however, that Skahen and Green (1948) found increased antidiuretic material in urine after treatment with DCA.

TABLE 6. SHOWING THE EFFECT OF SALINE AND OF ADRENAL PREPARATIONS ON THE SERUM ADS LEVEL OF INTACT RATS

Treatment	Number test animals	Percentage water excreted at 90 min.
Cortical extract 10 ml. per day, 5 days.	5	29.0
DCA, 5.0 mg. (in oil) per day, 5 days.	4	39.8
2 (15 mg.) DCA pellets for 3 weeks.[1]	6	36.2
2 (15 mg.) DCA pellets for 5 weeks.[2]	5	34.7
6 (15 mg.) DCA pellets for 6 weeks.[3]	5	36.0
10 (15 mg.) DCA pellets for 7 weeks[4] plus saline drinking fluid for last 4 days.	3	30.0
Saline (1.0%) drinking fluid for 7 days.	12	38.3

[1] 0.29 mg. absorbed per day.
[2] 0.37 mg. absorbed per day.
[3] 0.86 mg. absorbed per day.
[4] 1.06 mg. absorbed per day.

Sensitivity of adrenalectomized animals to ADH. An attempt was made to determine whether adrenalectomized animals are more sensitive to antidiuretic influences than intact animals. It has been previously demonstrated that for the first 18 hours following adrenalectomy rats show a normal diuretic response to small (5 ml. per 100 gms.) doses of water but

not to larger ones (Gaunt, 1946). It was found that 5 milliunits of Pitressin was more effective (P = less than 0.001 at 90 minutes) in reducing the rate of water excretion in animals adrenalectomized for 18 hours than in sham-operated controls (Figure 3). Thus it becomes apparent that in addition to the accumulation of antidiuretic material in the blood after adrenalectomy, there is also an increased sensitivity to antidiuretic agents. These results are consistent with those of Corey and Britton (1941) obtained under different circumstances.

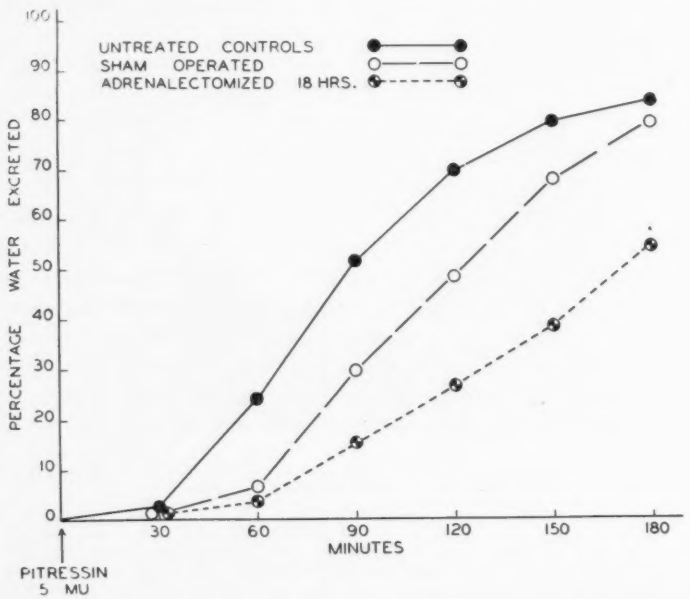

FIG. 3. Showing the effect of Pitressin on the excretion of administered water by rats adrenalectomized for 18 hours as compared with sham operated controls.

DISCUSSION

Most studies regarding the rate of secretion or the effective circulating amounts of posterior pituitary hormone have been based upon indirect evidence, such as: (1) The accumulation of antidiuretic material in the urine under conditions demanding maximal conservation of water (Gilman and Goodman, 1937). (2) Alterations in the rate of urine flow in heart-lung-kidney as compared with head-heart-lung-kidney preparations (Verney, 1926). (3) Restoration of renal function in diabetes insipidus animals by use of posterior pituitary extracts (Shannon, 1942). While many attempts have been made to demonstrate the presence of antidiuretic

material in the blood (Marx, 1930; Anselmino *et al.*, 1932; Marx and Schneider, 1934; Melville, 1937; Gilman and Kidd, 1938; Walker, 1939) by various assay methods the results have been, in general, inconclusive or controversial.

While few would doubt that the main humoral agent concerned in water conservation in mammals is the neurohypophyseal ADH, the accessory role of other factors is far from clear. Antidiuretic substances of hepatic origin have been described by Theobald and White (1933); Schaffer *et al.*, (1941); Baez *et al.*, (1949). The latter workers have identified the hepatic antidiuretic substance (VDM) as a definite compound, ferritin. Antidiuretic substances derived from kidney tissue have been reported by Pickering and Prinzmetal (1940). More recently it has been reported (Zarrow, 1949) that the hormone-like substance relaxin, present in blood serum and other tissues, causes water retention in rabbits. Studies on the toxemias of pregnancy have suggested the accumulation in body fluids of antidiuretic substances whose identity and origin is still uncertain. While we report here an increase in antidiuretic material in blood after adrenalectomy, a substance not decreased by overdosage with cortical hormone, Skahen and Green (1948) found that DCA or salt would cause the appearance of an antidiuretic agent in the urine of intact rats developing polyuria and hypertension.

Question has been raised regarding the site of origin of the antidiuretic materials which have been reported in urine. While most workers consider them to be of pituitary origin (Gilman and Goodman, 1937; Ralli *et al.*, 1945) others have objected on the grounds that such material could be detected in hypophysectomized animals (Walker, 1939) or that the urinary substances were not identical to known pituitary preparations (Ham and Landis, 1942). While the former objections are as yet unexplained, recent studies of Grollman and Woods (1949) indicate the possibility that oxidative alterations could account for the differences between the urinary antidiuretic substances and posterior pituitary extract. The antidiuretic material found in urine in cases of liver dysfunction might be antidiuretics of hepatic origin, or substances of hepatic origin which work by stimulating production of posterior pituitary ADH, or the latter hormone itself accumulating because it is not inactivated normally. Considerable evidence is available which can best be interpreted as indicating that in certain types of hepatic disease antidiuretic materials are inactivated at subnormal rates.

It is probable, therefore, that the humoral agents affecting water metabolism are numerous and their interactions devious. In view of this confusing variety of antidiuretic agents in body fluids and tissues, the positive claim that the substance in serum which we have described is

circulating posterior pituitary hormone would be premature. The observation that this material gives reactions similar to posterior pituitary extracts in all tests to which it has been subjected, however, plus the absence of detectable serum ADS in hypophysectomized rats, obviously suggests its pituitary origin. The detection of a somewhat similar material in the blood of man under a variety of clinical conditions (Lloyd and Lobotsky, 1949a; 1949b) and the alterations observed in serum ADS levels of rats subjected to hydration and dehydration (Jenkins and Birnie, 1949) are best explained on the theory that the antidiuretic material is of pituitary origin.

We noted the presence of this ADS in blood serum while seeking the cause of failure of the diuretic response to administered water in adrenalectomized rats. The observed increase of the antidiuretic substance following the removal of the adrenals is in harmony with the report of Martin *et al.* (1939) that an antidiuretic substance appears in the urine of cats after adrenalectomy. Knowledge that such a substance accumulates aids in explaining the alterations in salt and water metabolism which characterize adrenal insufficiency (Gaunt, Birnie and Eversole, 1949).

The mechanism responsible for the accumulation of the ADS after adrenalectomy is as yet unknown, but one possible explanation has appeared in other work (Eversole *et al.*, 1949). Pitressin and serum ADS are inactivated *in vitro* by a cell-free liver extract (enzyme). The ability of liver extracts to inactivate posterior pituitary preparations is decreased after adrenalectomy (Birnie, unpublished). Therefore, it may be that an ADS accumulates after adrenalectomy because of decreased destruction rather than over-production.

Antidiuretic materials in serum of other species. In preliminary observations, using the rat test described here, we have observed antidiuretic activity without exception in 1 ml. samples of serum from six hamsters and three dogs. It has also been true, again without exception, that this activity was less than that exhibited by rat serum. Lloyd and co-workers also find normal human serum, tested in rats, less antidiuretic than rat serum. The human material in addition apparently differs from that of rats in being less labile. This may mean either that the effective agents in the two species differ or that the inactivating systems differ. At least, the rat is likely unusual in having a particularly high titer of antidiuretic material in its serum.

SUMMARY

Normal rat blood serum contains a substance(s) with the following properties. It is antidiuretic, chloruretic, inactivated by standing, and more active by intraperitoneal than subcutaneous injection; it is not dependent

on ether anesthesia for its presence and is inactivated by neutralized thioglycollic acid. It exerts its antidiuretic action by causing an increased renal tubular reabsorption of water despite causing a transient rise in the glomerular filtration rate. Its effects are not duplicated by foreign proteins. It is not present in the blood of hypophysectomized rats and in all respects tested its actions are like those of posterior pituitary extract. Its pituitary origin is suggested but not regarded as established. There is evidence that a similar substance is present in the blood of other species but perhaps in lower concentration.

The substance accumulates after adrenalectomy, and this fact plus the demonstrated hypersensitivity to antidiuretic agents helps to explain the absence of normal water diuresis after adrenalectomy. The accumulation of the antidiuretic substance after adrenalectomy is readily prevented by replacement therapy with saline, DCA or adrenal cortical extract. Overdosage with cortical hormones, however, does not reduce the amounts present in the blood of normal rats.

A method (modified from Heller and Urban) is described for tests of antidiuretic substances.

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AN IMMEDIATE RESPONSE OF THE DEMEDULLATED ADRENAL GLAND TO STRESS

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INTRODUCTION

THE role of the pituitary and adrenal glands in the "alarm reaction" of Selye (1946) has been extensively investigated in recent years. However, the mechanism by which the corticotrophic activity of the pituitary is stimulated by various damaging agents is unknown.

It was pointed out by Long (1946) that the release of epinephrine was one activity associated with all of the various types of stress, and he advanced the hypothesis that epinephrine might be an essential agent in the process leading to the liberation of ACTH. Vogt (1944) was the first to present evidence that epinephrine could elicit an increased activity of the adrenal cortex. By assaying the corticoid activity of the adrenal venous blood of the dog she was able to demonstrate an increased production of cortical hormones following the administration of epinephrine. These findings were confirmed by Long and Fry (1945) who demonstrated that physiological doses of epinephrine produced a decrease in the cholesterol and ascorbic acid concentration of the adrenal glands of intact rats, but not in hypophysectomized rats. The discharge of cholesterol and ascorbic acid from the adrenal gland in response to a variety of agents has been shown (Sayers, Sayers, Liang and Long, 1945) to be a specific response to pituitary corticotrophin.

Gershberg and Long (1948) then showed that insulin also produced a decrease in the ascorbic acid concentration of the adrenal gland, but that this decrease did not occur if the hypoglycemia was prevented by a concomitant administration of glucose. Since it had previously been shown (Cannon, McIver and Bliss, 1924) that insulin hypoglycemia causes release of epinephrine, and since "glucose does not prevent activation of the pituitary in such situations as exposure to cold or trauma, where the release of epinephrine is due to cause other than hypoglycemia," these authors concluded that "it is not the level of blood glucose itself but the release of epinephrine that determines the activity of the pituitary."

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Although the tentative hypothesis, that the release of epinephrine is an integral part of the mechanism activating the pituitary-adrenal axis, is compatible with these observed phenomena, the evidence is all indirect, and there has been little or no direct approach to this problem. It was the author's contention that the rôle of the adrenal medulla in the corticotrophic response to stress could best be evaluated by measuring this response in the adrenal-demedullated animal, and it was for this purpose that the present investigation was undertaken.

The most reliable method for obtaining a complete demedullation of the adrenal gland (Griffith and Farris, 1949) is the one described by Evans (1936). The entire adrenal gland is enucleated, leaving only the capsule. As there is no regeneration of medullary tissue, the regenerated adrenal gland consists entirely of cortical tissue. In order to measure corticotrophic activity by changes in the ascorbic acid concentration of such a regenerated, demedullated gland it was first necessary to learn at what time after demedullation the ascorbic acid concentration of such a gland becomes constant. It was next necessary to establish that the demedullated gland responds to corticotrophic stimulation with a discharge of ascorbic acid, by demonstrating this discharge following the administration of exogenous epinephrine. Then one could determine whether the adrenal medulla is essential for the pituitary activity following exposure to stress by studying the effect of several stressing agents (i.e., cold, histamine, insulin) on the adrenal ascorbic acid concentration and eosinophil count of adrenal-demedullated animals.

METHODS

Ascorbic acid concentration of the demedullated adrenal gland

Female piebald rats weighing 80–110 gm. were divided into four groups of 6–8 rats each. Group I served as normal controls, while groups II, III, and IV were demedullated at 8 day intervals, by the method described by Evans (1936), and all animals were sacrificed at the same time: 32, 40, and 48 days after the demedullations. In addition one rat was sacrificed 12 days after demedullation and 2 others were sacrificed 24 days after demedulation.

All animals were fed Purina fox chow and tap water ad lib, except for a 5 day period following demedullation, during which time 1% saline was substituted for the tap water. The adrenals were removed and weighed individually, and the ascorbic acid concentration was determined by the method of Roe and Kuether (1943).

Response of the demedullated adrenal to stress

In the next experiment (table 2) female piebald rats weighing 60–100 gm. were demedullated in the same manner as above, and 35 days later, at which time they weighed 145–205 gm., they were divided into five groups of 6–7 animals. All animals were fasted so that the total duration of the fasting period was 17–18 hours before the time that they were sacrificed. Group I served as controls. Group II received 0.02 mg. epinephrine HCl/100 gm. body wt. subcutaneously and was sacrificed 2 hours later. Group III re-

ceived 0.02 I. U. insulin/100 gm. body wt. intraperitoneally and was sacrificed 1 hour later. This rather small dosage was employed because demedullated rats are quite sensitive to insulin (Gellhorn, Feldman and Allen, 1941). Groups IV and V received 1 mg. histamine HCl/100 gm. body wt. intraperitoneally, and were sacrificed at 1 and 4 hour intervals respectively.

The ascorbic acid concentration was measured in the right adrenal of each animal and the left adrenal was fixed in Bouin's solution, cut in 10μ sections, and every tenth section was stained with hematoxylin and eosin and examined microscopically for the presence of medullary tissue. The number of eosinophils/mm.^{3} of blood was counted in groups I and VI by Dunger's method as modified by Hills, Forsham and Finch (1948). The blood glucose concentration was determined by the macro-method of Folin (1929) in groups I and IV.

The third experiment (table 3) was performed on female piebald rats which were demedullated 42 days prior to the termination of the experiment. Group I consisted of 8 controls and group II consisted of 8 rats which were exposed to cold (0° C. $\pm$2°) for 1 hour, and sacrificed at the end of that time. Neither group was fasted.

TABLE 1. THE ASCORBIC ACID CONCENTRATION OF THE DEMEDULLATED ADRENAL GLAND

Group	I	II	III	IV
Number of animals	8	8	6	7
Days after demedullation	—	32	40	48
Adrenal weight (mg./100 gm. body wt.)	25.0 $\pm$ 0.17	18.0 $\pm$ 1.5	22.2 $\pm$ 2.2	19.8 $\pm$ 1.2
Adrenal ascorbic acid concentration (mg./100 gm. adrenal wt.)				
Left adrenal	445 $\pm$ 26	387 $\pm$ 29	361 $\pm$ 29	386 $\pm$ 25
Right adrenal	439 $\pm$ 25	378 $\pm$ 25	372 $\pm$ 26	370 $\pm$ 25
Combined adrenals[1]	442 $\pm$ 26	384 $\pm$ 27	366 $\pm$ 29	380 $\pm$ 23
Range of values (2 coefficients of variation)				
Left adrenal	34.1%	42.9%	39.6%	33.7%
Right adrenal	32.3%	37.0%	34.4%	35.7%
Combined adrenals	32.8%	39.6%	38.8%	31.3%

[1] Calculated by dividing the total amount of adrenal ascorbic acid by the total amount of adrenal tissue.

RESULTS AND DISCUSSION

Ascorbic acid concentration of the demedullated adrenal gland

The one animal killed 12 days after demedullation had no ascorbic acid in either adrenal gland. The two animals killed 24 days after demedullation had an average concentration of 267 mg. ascorbic acid/100 gm. adrenal weight. As can be seen in table 1, the total amount of adrenal tissue/100 gm. body wt. and the adrenal ascorbic acid concentration both become constant about the 32nd day following demedullation, although neither of these values is as great as those for the normal animal. This interval is the same as that reported by Greep and Deane (1949) who found, employing histochemical techniques, that 32 days after adrenal enucleation the "ascorbic acid had reappeared in all of the cortical cells in essentially normal amounts."

The range of values for the ascorbic acid concentration, expressed as two coefficients of variation, is about the same 48 days after demedullation as in the control group, and is slightly greater at the 32 and 40 day periods. There is a greater disparity in the size of the two adrenal glands of any one animal in the demedullated groups than in the control group, as 67% of the demedullated animals had a difference of more than 5 mg. in the weights of their two adrenals while only 25% of the controls showed this great a difference. The difference in the ascorbic acid concentrations of the two glands of the same animal was also greater in the demedullated animals (32 mg.%) than in the control group (11 mg.%). However, there was no

TABLE 2. THE EFFECT OF VARIOUS AGENTS ON THE DEMEDULLATED ADRENAL GLAND

Group	No. animals	Treatment	Duration (hrs.)	Adrenal ascorbic acid (mg./100 gm. adrenal)	% decrease	Eosinophils (per mm³ blood)	Blood sugar (mg. %)
I	6	Control	—	328 ± 34	—	101 ± 24	68 ± 4
II	6	Epinephrine	2	239 ± 12	27%		
III	7	Insulin	1	209 ± 8	36%		53 ± 1.5
IV	7	Histamine	1	215 ± 14	34%		
V	6	Histamine	4	206 ± 18	37%	38 ± 7	

significant difference in the ascorbic acid concentration of the large adrenals (400 ± 21 mg.%) and the small adrenals (383 ± 16 mg.%) of those demedullated animals with a difference of more than 5 mg. in the weights of their two adrenals ($p = <.6$), so the differences in weight and ascorbic acid concentration are apparently unrelated. Moreover, the average difference in the concentration of ascorbic acid in the two glands of these animals (32 ± 7 mg.%) is the same as that found in the demedullated animals with a difference of less than 5 mg. in the weights of their two adrenals (32 ± 9 mg.%).

It was thus felt that the ascorbic acid concentration and range of values which we obtain in the demedullated adrenal gland 32 days after demedullation make such a preparation suitable for a quantitative study of the changes which occur in the adrenal ascorbic acid concentration. Furthermore, those animals with an unequal regeneration of their two adrenal glands are also suitable for this type of study, and in such instances either gland can be used for the determination.

Response of the demedullated adrenal to stress

The results are presented in tables 2 and 3. It can be seen that epinephrine, insulin, histamine and cold evoked a discharge of adrenal ascorbic acid, indicative of an increased secretion of pituitary adrenocorticotrophin.

Histamine produced an eosinopenia of 38 eosinophils/mm.^{3} of blood as compared to the control value of 101, providing evidence of an increased secretion of cortical hormones (Hills, Forsham and Finch, 1948).

The blood sugar determinations show that insulin produced the expected hypoglycemia.

Examination of the microscopic sections failed to demonstrate any remaining medullary tissue.

The ability of insulin, histamine and cold to stimulate an increased production of ACTH in demedullated animals demonstrates that the secretion of epinephrine from the adrenal medulla is not essential for the

TABLE 3. THE EFFECT OF COLD ON THE ASCORBIC ACID CONCENTRATION OF THE DEMEDULLATED ADRENAL GLAND

Group	No. animals	Treatment	Adrenal ascrobic acid (mg./100 gm.)	% decrease
I	8	Control	473 ± 28	—
II	8	Cold—1 hr.	325 ± 19	31%

increased pituitary activity following stress, nor is it, as Gerschberg and Long (1948) contend, the agent responsible for the pituitary activity following insulin hypoglycemia.

This investigation has failed to contribute any positive information concerning the mechanism by which the anterior pituitary gland is activated by damaging agents, but it does exclude the adrenal medulla as a necessary component of such a system, and confirms the original contention of Selye (1946, p. 183) that the chromaffin system plays a prominent rôle only during the first few minutes of the alarm reaction and that "it is questionable whether it is of great importance at any other time during the course of the general adaption syndrome."

SUMMARY

The ascorbic acid concentration of the regenerated, demedullated adrenal gland of the female rat becomes constant about 32 days following enucleation.

Insulin, histamine and exposure to cold produce a marked decrease in the concentration of ascorbic acid in the demedullated adrenal gland of the rat in one hour.

Histamine produces an eosinopenia four hours after administration in the adrenal-demedullated rat.

It is therefore concluded that the adrenal medulla is not essential for the activation of the pituitary gland by stressing agents.

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RENOTROPIC EFFECTS OF SOME ANDROGENS UPON EXPERIMENTAL HYDRONEPHROSIS AND UPON THE CLITORIS IN THE MOUSE[1]

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THE growth-stimulating effects of androgenic steroids upon the renal parenchyma and their influence upon some of its metabolic functions have long been known and have recently been reviewed by Henderson (1949).

Renotropic effects of androgens, particularly those of testosterone propionate upon the diseased kidney in experimental hydronephrosis (Selye, 1940), sublimate poisoning (Selye, 1941) and in man in the uremia of cholera (Henderson, 1949) have been described.

The present study was undertaken in an attempt to utilize the property of the experimental hydronephrotic kidney of being susceptible to "protection" by steroid hormones as a simple means to evaluate the relative renotropic potency of such compounds. Simultaneously, effects of some androgens upon the weights of other organs in intact animals were investigated. A good correlation between dosage and effect was found to exist between testosterone and the weight of the clitoris. It is thought that this may furnish a simultaneous measurement of the androgenic effects or at least of the clitoritropic effect of compounds studied for renotropic activity.

METHODS

Experiments were designed to study the effects of various steroids in oil solutions upon organ weights and structure in mice.

The following substances[2] were used: Peanut oil U.S.P., cis testosterone, vinyl testosterone, methylandrostenediol, free testosterone, testosterone propionate, methylandrostanediol, testosterone-β-diethylaminoethyl carbonate and testosterone tocaptate.

These androgens were dissolved in sterile peanut oil U.S.P. in concentrations of 5 mg.

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[2] The steroids used in this study were provided by the Ciba Pharmaceutical Products, Inc., Summit, N. J., The Organon Inc. Orange, N. J., The Schering Corp., Bloomfield, N. J. and Winthrop Stearns Inc., New York.

of the steroid per 1 cc., except for the studies on dose-effect relationships, where the concentrations were modified to suit the particular experiments. Water soluble preparations were used as received from the manufacturers and the concentration of the steroid was adjusted to 5 mg./cc. by the addition of saline to more concentrated solutions.

Adult female Swiss white mice bred in our mouse colony from stocks obtained from the Jackson Memorial Laboratories in Bar Harbor were used. Purina chow and water were given *ad libitum* throughout the experiment. The mice were kept in an air-conditioned animal room at a temperature of 72° F and a relative humidity of 55%, and were weighed every other day.

In a first set of experiments, 5 groups of 10 mice received testosterone propionate in doses varying from 0.1 to 5 mg. by subcutaneous injection. Injections were given every other day over a period of 14 days. One additional group was given pure peanut oil. At the end of 14 days, the animals were killed by severing the cervical vertebral column; dissection was carried out at high humidity and the organs were weighed immediately.

In a second group of experiments, the left ureter was ligated just below the renal pelvis through an incision along the posterior costal margin under light ether anesthesia. On the following day, injections of 2.5 mg. of the steroids was begun. These were given subcutaneously every other day for periods varying from 30 to 40 days. Each steroid was given to a group of from 5 to 10 mice. Each experiment was controlled by a group of animals undergoing ureteral ligation alone and another group undergoing sham operations and given injections of pure peanut oil. At the end of the experiment, the animals were killed and dissected as in the previous experiment.

All tissues were fixed in 10% formalin, and hematoxylin-eosin preparations were made of the kidneys for the present study. The histology was studied on coded slides and the preparations were graded from 1+ to 4+ according to the degree of preservation of tubular epithelial cells which were found to survive within the fibrotic tissue replacing the renal parenchyma following the ureteral ligation.

RESULTS

1. *Effects of testosterone propionate on healthy kidneys in intact animals.* Weight curves (Figure 1) relating various dosages to renal weight showed a marked increase in weight of the kidneys at dosages beginning at 0.1 mg. every other day for 14 days (35 mg./kg.) and a maximal response at doses of 2.5 mg. every other day for 14 days or a total dose of 875 mg./kg. These doses per kg. body weight represent the total dose based on the average body weight of the animals in each experimental group.

2. *Effects of testosterone propionate on healthy kidneys in female animals with contralateral hydronephrosis.* Figure 1 demonstrates that the considerable contralateral compensatory renal hypertrophy is further increased by administration of testosterone propionate in much the same fashion as that observed in the normal kidney.

3. *Effects of various steroids on healthy and hydronephrotic kidneys in the same female animal.* In Figure 2 the compounds studied have been arranged in order of increasing renotropic effect as expressed by the weight increase of the contralateral healthy kidney in hydronephrotic mice. Weights of hydronephrotic kidneys were too widely scattered to be of any significance. The total doses used were 175 mg./kg. and 350 mg./kg.

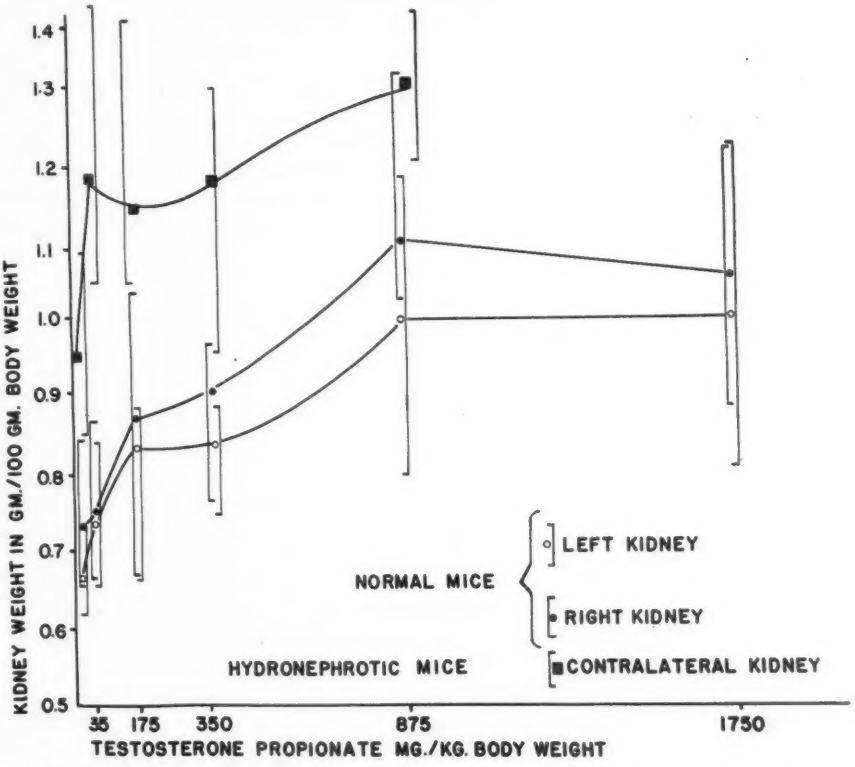

FIG. 1. Effects of increasing doses of testosterone propionate upon the weight of kidneys in normal female mice (two bottom curves) and upon the healthy contralateral kidney in mice having experimental hydronephrosis on the opposite side (top curve).

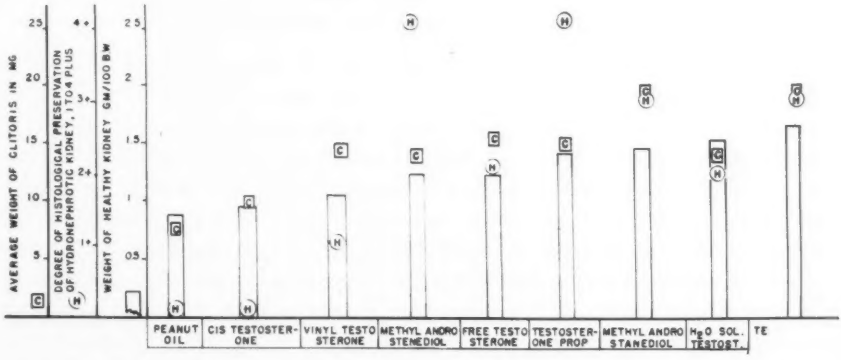

FIG. 2. Effects of 8 derivatives of testosterone and of peanut oil upon size of clitoris, histology of hydronephrosis and weight of healthy contralateral kidney.

The weight change of the healthy kidney does not parallel the degree of histological preservation observed in the hydronephrotic kidney in the same animal, although there may be some degree of correlation between the two factors.

The ligation of the one ureter in the mouse for at least 25 days causes extensive fibrosis of the corresponding renal parenchyma, which is reduced to a capsule a few mm. in thickness. In the control animal receiving peanut oil hardly a remnant remains of the tubular elements of the kidney. There

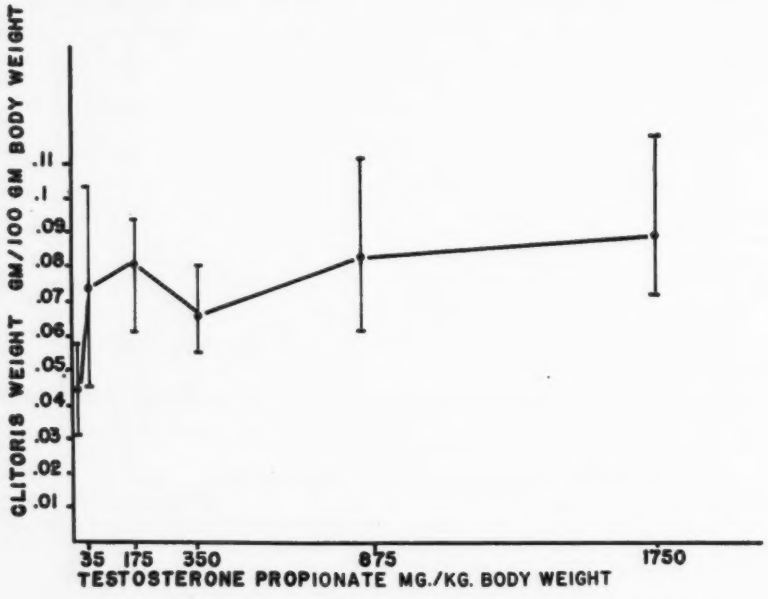

FIG. 3. Effects of increasing doses of testosterone propionate upon the weight of the clitoris.

are foci of round cell infiltration and scattered glomeruli with their surrounding Bowman's capsules markedly distended.

In those animals treated with androgens the kidneys with 4 plus protection show islands of tubular epithelial cells amidst the fibrotic tissue. These cells are huge and have a dark staining protoplasm, resembling hypertrophied tubular cells seen in the non-hydronephrotic kidney when stimulated by renotropic agents. Sometimes they are grouped in the fashion of renal tubules, while often forming irregular nests. Some sections show multinucleated cells, indicative of actual proliferation. The grading was done according to the relative predominance of such renal parenchymá tissue as compared with the fibrosis.

4. *Effects of testosterone propionate on the clitoris and the clitoral glands of the same animals.* These organs were used as suggested by Burdick and Gamon (1941). Increases in weight of the clitoris and of the clitoral glands with increasing doses of testosterone propionate become evident at 35 mg./kg. and maximal at 175 mg./kg. At 350 mg./kg. there was less effect and a slight additional rise occurred with 875 mg./kg. The scattering of these figures was reasonably narrow (Figures 3 and 4).[3]

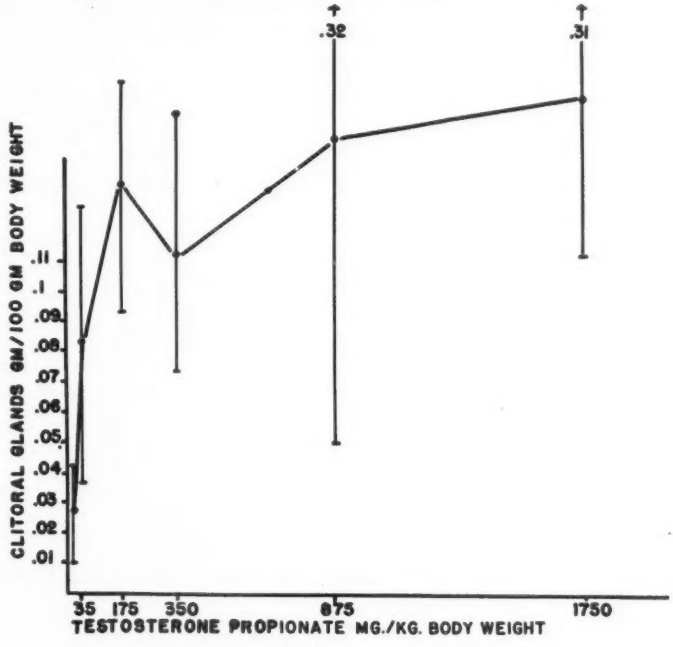

FIG. 4. Effects of increasing doses of testosterone propionate upon the weight of the clitoral glands.

5. *Effects of various steroids on the clitoris of female mice.* The control weights of the clitorides of mice receiving peanut oil were 6.83 mg. to 12.5 mg. The clitoral weights obtained in mice receiving testosterone propionate (250 mg./kg.) average 14.6 mg. and those of the other groups are given in Figure 2.

DISCUSSION

A glance at Figure 2 shows that the increase in weight of the normal

[3] Statistical evaluation by Dr. J. W. Youden, National Bureau of Standards, Consultant Biometrician of the Cancer Research and Cancer Control Unit confirmed the significance of these data.

kidney parallels closely the degree of "androgenicity" of the compounds tested as expressed by the weights of the clitoris. The only exception occurs in the case of the water soluble preparation where the renotropic effects exceed the clitoritropic action as might be expected because of the rapid excretion of the water soluble testosterone through the kidney.

The protective effect of the steroids upon the tubular epithelium of the hydronephrotic kidney does not parallel the classical renotropic effect. In the case of vinyl testosterone and cis testosterone, the effect upon the clitoris is more marked than the renotropic effect, whereas with testosterone propionate, methylandrostanediol and, to a slight degree, methylandrostanediol, the effect upon the diseased kidney exceeds the action upon the clitoris as well as the renotropic effect upon the healthy kidney. This relationship exists regardless of whether the renotropic effect upon the healthy kidney be measured by weight or by the degree of epithelialization of Bowman's capsules.

It may thus be postulated that the effects upon the experimental hydronephrotic kidney measure properties of steroid hormones which are different from those causing enlargement of healthy kidneys.

The possibility exists that these new properties may have therapeutic value in man, since they are so pronounced in testosterone propionate which is the only steroid agent known so far to have therapeutic properties in cancer of the breast, as a protein anabolic hormone and a therapeutic renotropic substance in man. It is suggested that the use of the hydronephrotic kidney may be a helpful screening test to discover some new therapeutic agents among steroid hormones. Clinical experience obtained with methylandrostenediol in our laboratories supports this view and has been published elsewhere (Homburger, Kasdon and Fishman, 1950).

SUMMARY

Results are reported of bioassays in mice of androgenic steroid hormones, using their renotropic effects upon the healthy kidney and in experimental hydronephrosis, as well as their effect upon the weight of the clitoris.

It was found that the effects upon the healthy clitoris and upon the healthy kidney were usually of the same order of magnitude in the substances tested.

In some instances, the effect upon the tubular epithelium of the hydronephrotic kidney falls out of line. It is particularly increased in the case of testosterone and methylandrostenediol.

It is postulated that the degree of protection afforded tubular epithelial cells of the hydronephrotic kidney expresses properties of steroid hormones which differ from the classical renotropic effect. This may serve to detect

steroids of testosterone-like properties in the screening of compounds of unknown biological activity.

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THE GLYCOSTATIC ACTION OF PURIFIED GROWTH HORMONE[1]

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WHEN food is withheld from hypophysectomized rats, the carbohydrate stores of the body become depleted more rapidly than in normal rats. The liver glycogen and blood glucose begin to fall within a few hours, and later the glycogen content of muscle (gastrocnemius) declines to levels well below those seen in normal rats even after prolonged fasting (Russell and Bennett, 1937). During a 24-hour fast, the muscle glycogen can be maintained at normal values or above by treatment through this time with alkaline anterior pituitary extracts, an action termed a "glycostatic" effect (Russell and Bennett, 1936; Russell, 1938). This effect is independent of the adrenal cortex (Bennett, 1938; Bennett and Perkins, 1945). In early tests of a variety of pituitary fractions (Russell, 1938), crude unfractioned extracts were found to be most active, and no relationship was indicated between the glycostatic factor and any of the known anterior lobe hormones. The minimum effective dose of organic material in the alkaline extracts was of the order of 1 mg. per 100 gm. body weight. Later, Herring and Evans (1943) were able to test more highly concentrated pituitary fractions. When given to hypophysectomized rats during a 24-hour fast, purified adrenocorticotrophic and lactogenic hormones and a partially concentrated thyrotrophic fraction were inactive at doses of 2 to 3 mg. per 100 gm. Partially purified growth hormone preparations, on the other hand, were usually effective in maintaining muscle glycogen levels when given in amounts of 0.1 to 0.5 mg. per 100 gm., although this degree of activity was not observed in every series. We have now found that highly purified growth hormone prepared by the method of Wilhelmi, Fishman, and Russell (1948) is effective in maintaining the fasting muscle glycogen levels in hypophysectomized rats.

METHODS

Young adult male rats of the Sprague-Dawley strain were employed in these experiments. Most of the animals were hypophysectomized at the age of 2½ to 3 months (body

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[1] This investigation was supported by a Grant-in-Aid from the American Cancer Society, on recommendation of the Committee on Growth of the National Research Council.

weight 200–250 gm.). Complete atrophy of the gonads and accessory organs was accepted as indicating complete removal of the anterior lobe. Observations were made between 2 weeks and 3 months after operation; no differences in response were observed which could be attributed to varying time intervals between operation and treatment. The diet consisted of Purina Laboratory Chow, on which hypophysectomized rats in this laboratory survive indefinitely. The animals were maintained both before and after operation at an atmospheric temperature of 24–25° C.

Several different growth hormone preparations were employed. Those designated as semi-purified, crystalline, were prepared as described by Fishman, Wilhelmi, and Russell (1947). They were highly active in promoting growth but by electrophoretic analysis appeared to contain only about 60 per cent growth hormone. The purified preparations (Wilhelmi, *et al.*, 1948) appeared homogenous by electrophoretic and by ultracentrifugal examination. The hormone preparations were preserved in the dry state *in vacuo* at room temperature; for use, they were taken up in 0.9 per cent NaCl, with the addition of a small amount of 0.1 N NaOH if needed to effect solution. The animals were given the hormone preparations intraperitoneally in 2 equal doses, at the beginning of the 24-hour fasting period and again 8–10 hours later. The volume of solution injected per dose was usually 0.1 ml. per 100 gm. body weight.

At the end of the experimental period, the animals were anesthetized with sodium pentobarbital (Nembutal, Abbott), 5 mg. per 100 gm. The two gastrocnemii were removed and placed in tared tubes containing 30 per cent KOH. Glycogen was precipitated from the hot alkaline solution with 2 volumes of ethyl alcohol, and after standing overnight in the cold, hydrolyzed in hot 1 N acid for 3 hours. Glucose was determined with the phosphate-buffered copper reagent of Somogyi (1945) and Nelson's color reagent (1944). The results (average of the values from the two muscle samples) were expressed as mg. of glycogen per 100 gm. wet weight of tissue. The concentration of glycogen seen in normal rats under these conditions is about 520 mg. per cent.

RESULTS

The effects of treatment with growth hormone on the fasting muscle glycogen of adult hypophysectomized rats are summarized in Table 1. It is apparent that highly purified growth hormone is an effective glycostatic agent. The minimum effective dose (total amount given during the fast) under the conditions described above is of the order of 0.1 to 0.2 mg. per 100 gm. body weight.

In addition to the results presented in Table 1, observations were made also on rats which had been hypophysectomized at a younger age (body weight 90–100 gm.). Without treatment the muscle glycogen of such animals is rather lower than that of larger rats, usually about 300 mg. per cent. When growth hormone was given as described above, little effect on the muscle glycogen levels was seen with total doses of 0.2 to 0.5 mg. per 100 gm. However, when the hormone was given more frequently (at 24, 18, 12, and 6 hours before sampling) partial maintenance of the muscle glycogen was effected. In the data of Marx and Evans, most of the instances in which their growth hormone preparations failed to maintain muscle glycogen were those in which the test animals were female rats

hypophysectomized at 26–28 days of age, while maintenance was effected when the same fractions were given to male rats operated at 2 to 3 months of age. It appears that the smaller animals are less sensitive to the glycostatic action of growth hormone. A possible explanation for this difference in sensitivity could be that the smaller animals have higher metabolic rates per unit body weight and so tend to metabolize their glycogen stores at higher rates; at present we have no data to support either this or alternative explanations.

EFFECT OF GROWTH HORMONE ON THE MUSCLE GLYCOGEN OF FASTING HYPOPHYSECTOMIZED RATS

	No. of animals	Dose of growth hormone* μg. per 100 gm.	Muscle glycogen (gastrocnemius) mg. per cent†		Comparison with untreated group	
			average	standard error	t‡	p
Untreated rats	8	—	403	± 9		
Semipurified hormone (crystalline)	5	100	475	±20	3.75	<.01
	4	200	551	±39	5.16	<.01
Purified hormone	3	50	383	±15	1.23	>.20
	4	100	401	± 3	0.16	>.90
	8	200	536	±25	5.10	<.01
	7	400–500	518	±24	4.83	<.01

* Total dose given in 2 portions, 24 and 14–16 hours before analysis.
† Average value in fasting intact rats, 520 ± 10 mg. per cent.
‡ By Fisher's method for small samples.

The earlier failure to identify the glycostatic factor with growth hormone (Russell, 1938) was probably due to the crudity of the preparations available at that time and to inaccuracies of the bioassay methods then in use. Most of the active fractions contained growth hormone, but some adrenotrophic fractions which appeared to have little growth hormone activity were also effective as glycostatic agents. It now seems likely that the high concentrations of adrenotrophic hormone in these fractions may have masked the presence of growth hormone, since the two factors are antagonistic with respect to growth (Marx, *et al.*, 1943).

SUMMARY

Highly purified pituitary growth hormone has been found to maintain normal concentrations of muscle glycogen in fasting hypophysectomized rats. The minimum effective dose, given during a 24-hour fasting period, is of the order of 0.1 to 0.2 mg. per 100 gm. body weight.

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THE ABILITY OF LIVER PREPARATIONS AND VITAMIN B_{12} TO MAINTAIN THYMUS WEIGHT IN THYROID-FED RATS HAVING GREATLY HYPERTROPHIED ADRENAL GLANDS

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IN AN effort to deplete laboratory animals of as yet unidentified nutritional factors and so obtain a suitable animal for assay Betheil (1947) and Ershoff (1947, 1948) have included desiccated thyroid gland or iodinated casein in their experimental diets. This device serves to increase the basal metabolic rate of the animals so treated (Ershoff [1947], Robblee [1948]) and so results in a greater demand for nutritional factors not available in adequate amounts either in the diet or by intestinal synthesis.

Elvehjem *et al.* (1947a, 1947b) observed that the feeding of a natural ration was more satisfactory than a synthetic diet for the assay of unknown factors in commercial liver preparations. They combined this ration with the feeding of desiccated thyroid to chicks and were able to obtain significant growth responses to the administration of liver.

Folley (1947) as well as other workers (Zucker [1948]) have observed the decreased growth rates obtained on the litters from animals bred on synthetic or otherwise deficient rations. This decreased growth rate is presumably due to a decreased natural reserve of some dietary factors not available to the mothers during gestation and consequently present in reduced quantities in the young.

The above three devices were combined to provide an animal suitable for the assay of as yet incompletely known and understood factors present in commercial liver extracts. The present report concerns itself with some incidental observations on the thymus and adrenal weights of animals so treated.

METHODS

The following diet was used throughout the course of these experiments: soy bean meal (44% protein), 60; dextrose, 24; salts IV (Hegsted, [1941]), 4; crisco (or other vegetable fat), 10; cod liver oil 2 gm. per 100 gr. and thiamin, 1; riboflavin, 2; pyridoxine, 1; d-Ca pantothenate, 10; nicotinamide, 10; inositol, 5; para amino benzoic acid, 30;

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choline, 100; biotin, 0.05; pteroylglutamic acid, 0.2; alpha tocopherol, 14.2; 2-methyl 1,4-naphthoquinone, 14.2 mg. per 100 gm. (Emerson, [1948]).

Male and female rats of the Wilson strain, weighing between 200 and 350 grams were mated and at the end of three weeks the females were placed in isolation cages. At the time of delivery all litters were reduced to eight in number. When the young were twenty-one days old they were weighed, divided into groups of equal numbers of males and females of average equal weight and treated as indicated in the tables to follow. From the time of mating all female rats and their litters were given *ad libitum* access to the above diet and to drinking water. When the young were divided into experimental groups this diet was continued in conjunction with the supplements indicated below. The various groups were caged separately and groups contained either four or six animals, the number in all groups being consistent for each experiment. In all cases the average initial group weight was between 35 and 45 grams.

When the animals received injections, these were given subcutaneously five days a week except in the final experiment. When supplements were given by mouth the amounts indicated were incorporated into the soy bean diet and fed in the usual manner. Each rat was weighed weekly and at the end of four, five, or six weeks, depending on the numbers remaining alive, they were killed with nembutal and the indicated tissues removed and placed in 10% formalin. These were later stripped of adhering fat and other extraneous tissue and weighed. In no case were tissues included from animals that had died spontaneously.

TABLE 1. ADRENAL AND THYMUS WEIGHT RESPONSES TO INJECTABLE LIVER IN RATS MAINTAINED ON A SOYBEAN DIET CONTAINING 0.25% DESICCATED THYROID

Treatment	Total no. of animals	Sex	Final body[3] weight range	Total weight gain[3] at 4, 5, or 6 weeks	Thymus[4] weight range mg./100 gm.	Total adrenal[4] tissue weight range mg./100 gm.
Soybean		11 M	149–151	105–113	108–306	6.9–31.5
Diet Controls	16	5 F			219 Ave.	16.6 Ave.
Soybean Diet+0.25%		13 M	82–105	35–69	34–229	23.4–42.4
dess. Thyroid[1]	19	6 F			96 Ave.	30.4 Ave.
Soybean Diet ±0.25%		10 M	130–133	86–96	105–301	19.1–43.1
dess. Thyroid+0.2–0.3 units[2] Injectable Liver	14	4 F			198 Ave.	30.6 Ave.
Soybean Diet+0.25%		8 M	96–107	60–60	78–191	18.7–35.4
dess. Thyroid+0.02–0.04 units Injectable Liver	10	2 F			129 Ave.	27.2 Ave.

[1] U. S. P. Thyroid (Wilson Laboratories).

[2] U. S. P. Units of anti-pernicious anemia activity. The clinical assay of Injectable Liver Extracts is not a precise procedure and consequently the difference between 0.2 and 0.3 units is not significant. The same is true for the following group. This allows 14 animals to be considered together in one group and 10 in the other group thus giving added significance to the figures obtained in individual experiments.

[3] The body weight figures listed here are the averages obtained in four different experiments and therefore the range is, in fact, the range of four averages.

[4] The thymus and adrenal weight range figures, however, are those of the highest and lowest individual tissues obtained from the total number of animals in the indicated groups.

RESULTS

Table 1 summarizes the results of four experiments, each containing four or six animals, and conducted approximately six weeks apart.

Examination of the tissue weight data shows that the groups which received the larger dosage of Injectable Liver had adrenal hypertrophy as great as the thyroid fed controls but at the same time the thymus did not show equal atrophy. Similarly, the animals receiving the lower dosage of liver have very great adrenal hypertrophy but have an intermediate thymus atrophy. This dosage is a little too low to show the optimum

TABLE 2. ADRENAL AND THYMUS WEIGHT RESPONSES TO VITAMIN B_{12} CONCENTRATE AND COBIONE IN RATS MAINTAINED ON A SOYBEAN DIET CONTAINING 0.25% DESICCATED THYROID

Treatment	Final body weight	Gain at 4 weeks	Thymus weight range mg./100 gm.	Total adrenal tissue weight range mg./100 gm.
Soybean Diet			102–129	10.1–17.0
Controls	118	84	169 Ave.	14.0 Ave.
Soybean Diet +0.25% dess. Thyroid	(5)	48	54–116	14.7–23.2
	82		71 Ave.	19.5 Ave.
Soybean Diet +0.25% dess. Thyroid +3%	123	76	54–157	17.2–33.8
Vitamin B_{12} Concentrate[1]			129 Ave.	27.1 Ave.
Soybean Diet +0.25% dess. Thyroid +0.1	115	82	98–217	15.7–21.8
μg. Cobione three times weekly			154 Ave.	19.6 Ave.
Soybean Diet +0.25% dess. Thyroid +0.01	(2)		86–95	16.3–19.2
μg. Cobione three times weekly	104	71	91 Ave.	18.5

All groups contained six male animals at the beginning of the experiment and the number remaining at the time of autopsy (4 weeks) is indicated by the figure in parenthesis where it is less than six.

[1] Experimental Vitamin B_{12} Mixture (Merck) #8R5704 containing 2 mg./lb. Vitamin B_{12}.

intermediate thymus weight but the average figure is in the correct direction.

The time of killing in each experiment was determined by the body weight and the number surviving in the thyroid treated control group and was chosen so as to have as many animals as possible for autopsy from this group. On this basis the duration of succeeding experiments became shorter and this was thought to be due to the fact that in later experiments some of the litters were obtained from breeders which had been bred on this diet for the second time and were, therefore, presumably more deficient in the factor(s) required by the litters to resist the effects of thyroid feeding. Body weight is, under these circumstances, a better criterion of the state of the animals than is the number of weeks of treatment.

Since the beginning of these experiments crystalline vitamin B_{12} (Cobione) had become available and Table 2 summarizes the results obtained

in response to its subcutaneous injection three times weekly (Monday, Wednesday and Friday) in the dosage indicated, as well as the figures from one group fed a vitamin B_{12} concentrate. Here, also, the same type of result was obtained. All thyroid treated animals have enlarged adrenals but where vitamin B_{12} concentrate or Cobione was given thymus atrophy was prevented in what appears to be a quantitative manner.

DISCUSSION

Tepperman, Engel and Long (1943) have reviewed the wide variety of circumstances in which hypertrophy of adrenal glands occurs in the animal body. Treatment with thyroxin is among the many types of damaging agents which are known to elicit this response. In view of the diversified nature of these damaging agents there is no reason to suppose that the persistent feeding of large doses of thyroxin or thyroid substance acts other than as any non-specific stress to the animal so treated. In reviewing older thyroid feeding experiments White (1947) suggests this. Therefore, in the present experiments the consistently large adrenals are considered to be a non-specific response to the damaging effect of persistent thyroid feeding.

Adrenal hypertrophy has usually been interpreted as a sign of greatly increased activity and although this may not always be true there is evidence that this is the case when thyroid-like material is the stimulus. Lowenstein *et al.* (1942) were able to show that thyroxin treated rats had a decreased resistance to intraperitoneal injections of potassium chloride at the height of the thyroxin treatment but after this had been discontinued and enough time had elapsed for its most severe effects to have worn off, but while the adrenals were still hypertrophied, these same rats had an *increased* resistance to injections of potassium chloride. The interpretation placed on this by Tepperman *et al.* (1943) is that "when the maximum thyroxin effect on the tissues had been withdrawn, the hypertrophied and now hyperfunctioning glands are able to meet the metabolic situation created by the toxic substance more effectively than are normal glands."

The functional relationship between the adrenal cortex and lymphoid tissue has been elucidated by Selye and co-workers (1946) and further elaborated upon by White and Dougherty (1946). In the presence of abnormal amounts of cortical hormone there is an atrophy of lymphoid structures. Examination of thymus weights on control thyroid-fed groups shows this atrophy to be a consistent finding in the present experiments. It is also further evidence for the increased production of cortical hormones by the enlarged adrenals.

Inspection of adrenal and thymus weight data on all thyroid-fed groups which received liver supplement or Vitamin B_{12} shows immediately that

while adrenal hypertrophy is just as great it is not accompanied by thymus atrophy equivalent to that present in the thyroid-fed groups receiving no supplement. Where dosage is adequate the thymus weights are as good as the soy bean diet controls.

Since large excesses of all the known vitamins were included in this diet it was unlikely that the quantities of liver supplement used were supplying known factors in amounts adequate to account for the observed effects. Several investigators have previously shown that deficiencies of various of the known B vitamins will cause enlargement of the adrenals. If the adrenal hypertrophy of the present experiments were due to a like deficiency then it would be expected that groups receiving liver supplements would show less adrenal enlargements. This is not the case.

Inspection of Table 2 shows that when Cobione is administered to thyroid fed animals in as small a quantity as 0.1 micrograms, three times weekly, it is possible to demonstrate a clearly distinguishable protective effect on thymus weights.

With the data in hand it is not our intention to suggest that Cobione is the only factor capable of protecting the thymus in this manner but simply to report the finding. It is hoped that further work will clarify its significance.

It is now well known that at least one of the functions of Vitamin B_{12} in bacterial metabolism is in the synthesis of thymine desoxyriboside (thymidine) (Wright [1948], Snell [1948], Shive [1948a, 1948b]). Leblond *et al.* (1948) have shown that the two areas containing the highest concentration of newly formed desoxyribonucleic acids are the thymus and the crypts of Lieberkühn. Whether these latter observations will provide the basis for an explanation of the protective effect of Cobione on thymus weight under the conditions described remains to be shown.

SUMMARY

Twenty-one day old rats bred on a ration containing soybean meal, dextrose, and vegetable fat, supplemented with excess amounts of all the crystalline vitamins were fed this diet plus 0.25% desiccated thyroid with and without supplements of Injectable Liver preparations, a Vitamin B_{12} concentrate, and Cobione. Graded growth responses were obtained with preparations of injectable liver extract and Cobione in four to six weeks.

All animals which received desiccated thyroid alone showed at autopsy greatly enlarged adrenals and thymus atrophy. Those animals which received liver preparations, the Vitamin B_{12} concentrate, and Cobione in addition to the desiccated thyroid had equally large adrenals but did not show a corresponding atrophy of the thymus. The protective effect on the

thymus weight of the various preparations used is quantitative under the conditions described.

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SOME EFFECTS OF THE HYPOPHYSIS ON IODINE METABOLISM BY THE THYROID GLAND OF THE RAT

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THE administration of thiouracil to the rat results in hypertrophy and hyperplasia of the thyroid gland and in decrease in thyroid iodine content, and these effects are dependent on the integrity of the pituitary gland (Astwood, 1944–45). Continued administration of thiouracil results in a state of hypothyroidism due to inhibition of thyroid hormone formation, and the thyroid gland changes are compensatory in nature, the result of increased elaboration of thyrotropic hormone by the pituitary. Thyroid glands rendered hyperplastic through the chronic administration of thiouracil are able to accumulate large amounts of iodine following injection of potassium iodide into the intact rat (Astwood, 1944–45), although this iodine can be retained for but a short time (VanderLaan and Bissell, 1946) and remains in ionic form (VanderLaan and VanderLaan, 1947). Thus, iodine can be concentrated by the thyroid gland despite the inhibition of its incorporation into thyroid hormone by the antithyroid agent. Further, under conditions of hyperplasia the capacity for iodide accumulation is greatly increased (VanderLaan and VanderLaan, 1947) and the efficiency with which a limited amount of circulatory iodide is utilized would thereby appear enhanced.

Astwood and Bissell (1944) investigated the influence of the hypophysis on iodine metabolism and found that hypophysectomy impaired reaccumulation of iodine in the thyroid gland previously depleted of iodine through thiouracil treatment. They further found that large doses of thyrotropin caused thyroid hyperplasia without appreciable loss of thyroid iodine. This paper presents additional evidence concerning the role of the hypophysis in the metabolism of iodine by the thyroid gland.

EXPERIMENTAL

50–100 gm., hooded, male rats were used. For long-term experiments with antithyroid medication, the animals were fed 0.03% or 0.1% propylthiouracil mixed with commercial Purina mink chow. Hypophysectomies were performed by the parapharyngeal approach. Thryotropin[1] was injected subcutaneously. Chemical determinations of iodine were made by the method of Astwood and Bissell (1944).

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[1] The thyrotropin was supplied through the kindness of Dr. D. McGinty of Parke, Davis and Company, Detroit, Michigan.

The thyroid/serum iodide ratio was determined by the use of radioactive iodine. Thirty to sixty minutes following a subcutaneous injection of 5 milligrams of an alkaline solution of propylthiouracil, a tracer dose of I^{131} was injected subcutaneously. One hour later, the animals were anesthetized with ether and blood was withdrawn by intracardiac puncture. The rats were then killed and the thyroid glands immediately dissected free of connective tissue and each lobe weighed separately. One lobe was used for determining the I^{131} present, the other was fixed for histological examination.

The lobe to be used for measurement of the radioactivity was placed on a thin piece of absorbent paper on a one-inch copper disc. The disc was then enclosed in Scotch tape and the gland pressed flat with a heavy weight. This flattening was accompanied by a drawing motion over the top of the Scotch tape to ensure a thin and even distribution of the tissue.

After centrifugation of the previously removed blood, 0.1 cc. of serum was pipetted onto a similar piece of paper covering a disc. After it had been allowed to dry, it was also covered with a single layer of Scotch tape. The discs were then counted by means of a mica-windowed Tracerlab Geiger-Muller tube. The I^{131}/100 mg. of thyroid tissue was then divided by the I^{131}/0.1 cc. of serum to give the thyroid/serum iodide ratio.

The ratio of cellular to non-cellular volume of the glands was determined by projecting representative sections 8 micra thick onto large sheets of drawing paper and tracing out the outer and inner cell borders of all acini. These were then cut out and weighed to give the ratio of cell-to-non-cellular volumes. Two representative areas were taken from each section, one from the center and one from the periphery, since the follicles tended to have larger lumina near the thyroid capsule. Two series of experiments were run; in one the glands were fixed in Bouin's solution and in the other in 10% formalin.

RESULTS

1. *The effect of hypophysectomy on the thyroid/serum iodide of glands made hyperplastic by long-term propylthiouracil administration.* In this experiment, the rats were first fed 0.03% propylthiouracil for two weeks or longer. Under these conditions, the thyroid-serum iodide ratio rises from the normal range of 25:1 to 200–300:1. Following hypophysectomy, although the animals were kept on the full dose of propylthiouracil, this ratio rapidly fell until the normal range had been reached in four to five days. The ratios in every case were above 160:1 before hypophysectomy, while two days after the operation all had fallen below this level. (See Figure 1.) When normal, untreated rats were hypophysectomized and immediately given propylthiouracil for eight to nine days, the ratio always dropped below 10:1 and frequently approached 1:1 (Greer, 1949).

The percentage of the cellular area of the glands was determined as described above. It was found that, although the cellular area tended to become slightly smaller with a decrease in the iodide-concentrating power of the thyroid, this correlation was so slight that it was considered insignificant. The data are presented in Figure 2.

It appears, therefore, that the iodide-concentrating mechanism of the thyroid is dependent on the presence of the pituitary. In hypophysec-

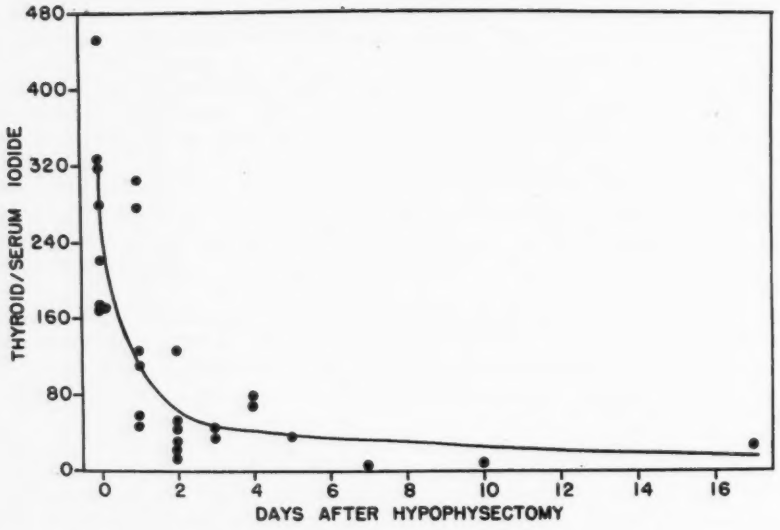

FIG. 1. Relation of thyroid: serum iodide ratio to the interval following hypophysectomy. Each point represents a single animal.

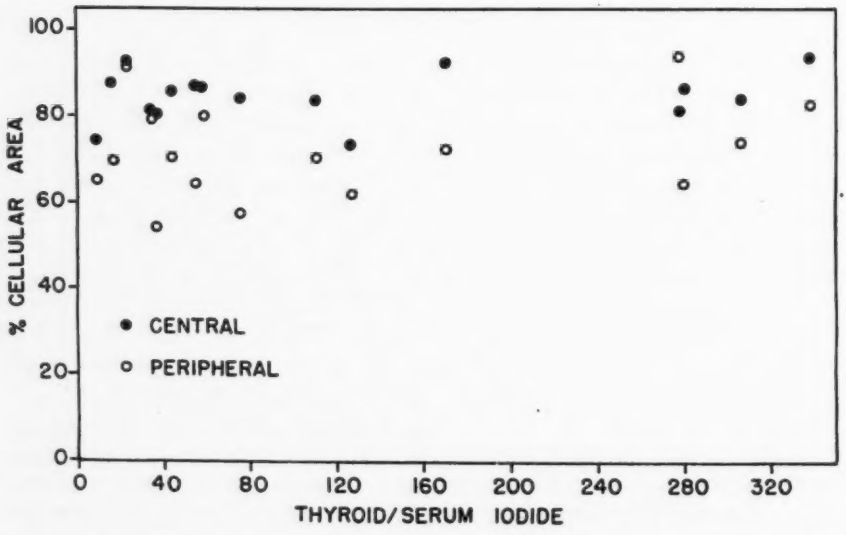

FIG. 2. Relation of thyroid: serum iodide ratio to cellularity of the thyroid. There is a very slight decrease in cellularity with a decrease in the iodide-concentrating power. Central and peripheral refer to the areas of the thyroid from which the projections were made.

tomized animals, the thyroid/serum iodide ratio started at high levels and rapidly fell to below normal although the thyroid remained anatomically hyperplastic for as long as ten days following pituitary extirpation. The thyroid weights also began to fall soon after hypophysectomy, but whereas in one week the weight had decreased to 50 per cent of the preoperative level (see Figure 3), the iodide-concentrating power had decreased to 1 per cent.

2. *The effect of hypophysectomy on the discharge of iodide from hyperplastic*

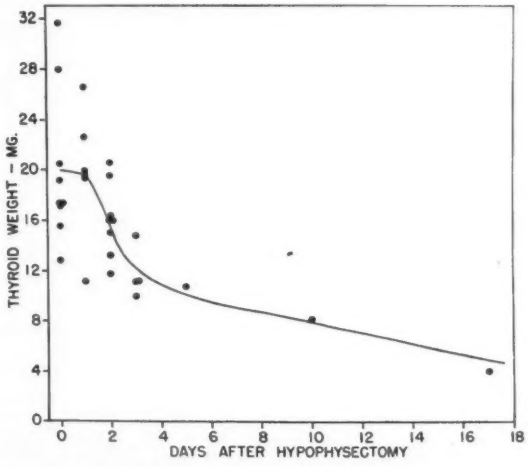

FIG. 3. Thyroid weights decrease most sharply between the first and third days following hypophysectomy. Thereafter the decline is more gradual.

glands. In this experiment the animals were also given propylthiouracil for more than two weeks before hypophysectomy. Twenty-four hours following the operation, 500 micrograms of potassium iodide were injected and the iodine content of the glands determined chemically at various intervals, propylthiouracil being continued. It can be seen from an examination of Figure 4 and Table 1 that the thyroids from rats which had been treated with propylthiouracil, but not hypophysectomized, reached a maximum iodide concentration at one to two hours. The thyroid iodide concentration then fell progressively as the iodide was excreted in the urine until 50 per cent had left the gland twenty-four hours after the potassium iodide injection. The hypophysectomized rats, on the other hand, lost the iodide from the thyroid much more slowly, three to four days having elapsed before the glands had lost 50 per cent of their maximal content. These data are hard to reconcile with the foregoing and are suggestive that dose differ-

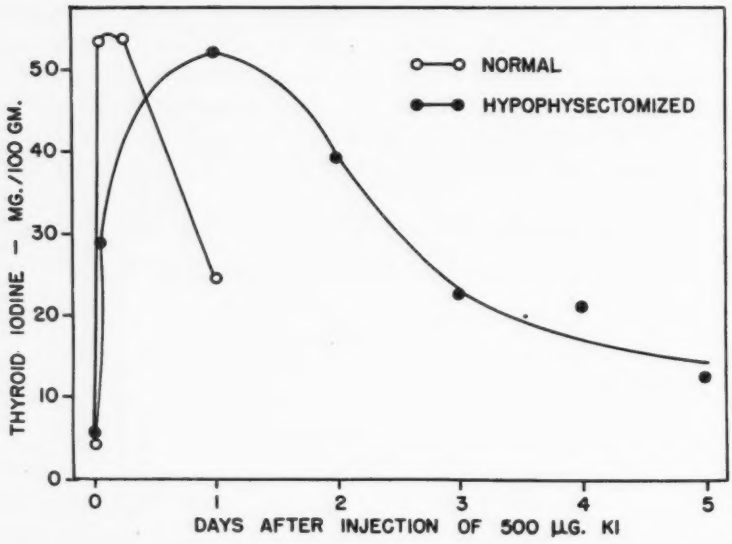

FIG. 4. The rapid uptake of iodine (iodide) by the thyroid gland of normal and of hypophysectomized rats fed propylthiouracil. In the former the iodide is retained for a short period; in hypophysectomized rats the iodide is slowly discharged.

ences are significant and that renal clearance of iodine may diminish after hypophysectomy.

3. *The effect of thyrotropin on the thyroid/serum iodide ratio.* This experiment was undertaken to determine whether injected thyrotropin would have the same effect on the iodide-concentrating mechanism of the thyroid as that from the animal's own pituitary. Untreated, intact, 50 gm. male rats were each injected with 20 mg. of thyrotropin containing 6–8 J-S units

TABLE 1. UPTAKE AND DISCHARGE OF IODIDE IN HYPOPHYSECTOMIZED AND NORMAL RATS BEING TREATED WITH PROPYLTHIOURACIL. 500 MICROGRAMS KI ONE DAY AFTER HYPOPHYSECTOMY

Time after 500γ KI s.c.	Days after hypophysectomy	Thyroid weight (average) mg.	Thyroid I_2 mg.%
No KI	1 d.	32.3	6.8
	1 d.	42.8	5.4
1 hr.	1 d.	28.8	28.7
24 hr.	2 d.	29.7	51.9
48 hr.	3 d.	27.8	39.6
72 hr.	4 d.	21.6	22.5
96 hr.	5 d.	23.2	21.0
120 hr.	6 d.	23.7	12.5
0 hr.	0	22.4	4.57
1 hr.	0	22.1	53.4
5 hr.	0	21.6	54.0
24 hr.	0	20.6	24.5

per milligram. The thyroid/serum iodide was determined at various intervals using a tracer dose of I^{131} as described above.

It can be seen that, although no stimulation was apparent at six hours, in twenty-six hours there was a 2-fold and in forty-eight hours a 3-fold increase in the iodide-concentrating power. By seventy-two hours, most of the effect of the thyrotropin had been dissipated and the ability to hold iodide had returned almost to normal. The data are presented in Figure 5.

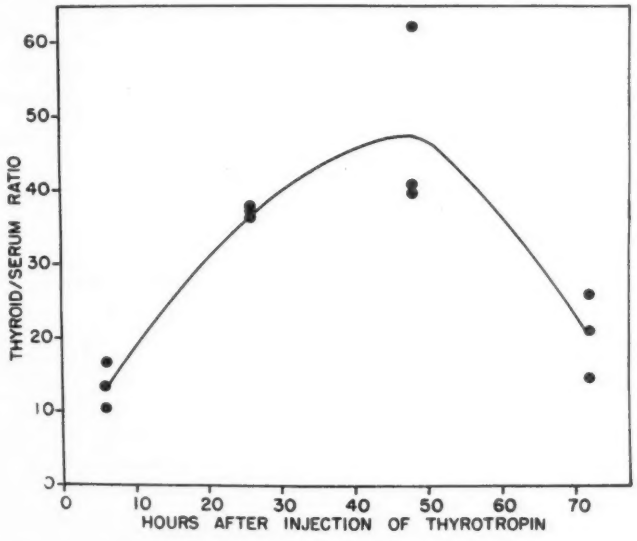

FIG. 5. The maximum increase in thyroid iodide-concentrating power occurs at 48 hours following a single injection of thyrotropin.

4. *The influence of the hypophysis on the formation of protein-bound iodine by the thyroid gland.* Propylthiouracil was given to rats for ten days or more to discharge all thyroid hormone from the gland and then was omitted from the diet. The pool of rats was divided into three groups. In the first group, after sampling to ensure that the thyroid iodine had been thoroughly depleted, the normal rate of reaccumulation of iodine was determined. At first the protein-bound iodine was separated by precipitation with zinc hydroxide, and later before the animals were killed a similar result was achieved by the injection of potassium thiocyanate to discharge any concentration of iodide ion from the gland. In the second group, hypophysectomy was performed at the time propylthiouracil was discontinued, and it was observed that for ten to fourteen days afterward little iodine was reaccumulated. This, in the light of other observations indicat-

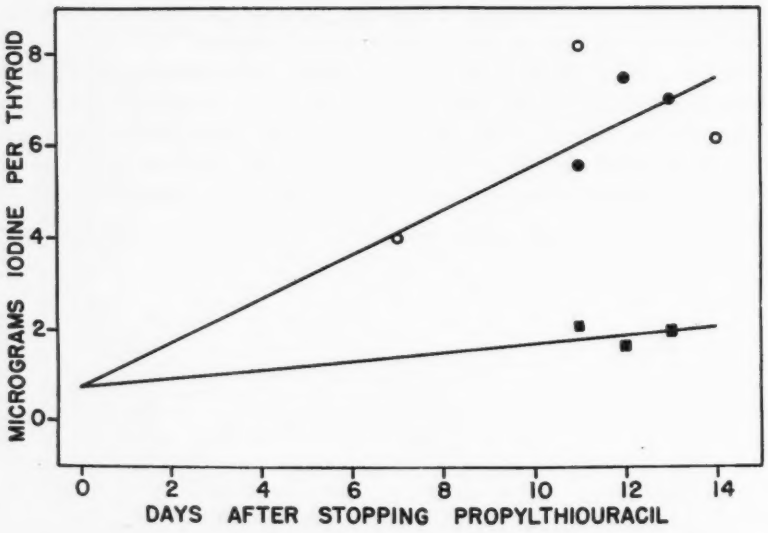

FIG. 6. The recovery of bound iodine in the thyroid glands of rats chronically treated with propylthiouracil.

○ Normal rats. ■ Hypophysectomized rats.

● Hypophysectomized rats injected daily with 5 mg. of thyrotropin.

The data indicate a rapid recovery of thyroid iodine in normal rats after discontinuing propylthiouracil in contrast to the minimal recovery by hypophysectomized rats at the time propylthiouracil is discontinued. Thyrotropin injection in the latter results in a normal rate of iodine recovery.

TABLE 2. THE RECOVERY OF "BOUND" IODINE IN THE THYROID GLANDS OF RATS. ALL RATS INITIALLY TREATED PROLONGEDLY WITH PROPYLTHIOURACIL. TOTAL THYROID IODINE CONTENT AFTER DISCONTINUING PROPYLTHIOURACIL IN NORMAL RATS, HYPOPHYSECTOMIZED RATS, AND HYPOPHYSECTOMIZED RATS SUBCUTANEOUSLY INJECTED WITH 5 MILLIGRAMS THYROTROPIN DAILY IS TABULATED

		No. rats	Average thyroid I_2 content	Days after propylthiouracil stopped
Group A	Controls	7	0.77 μg	0
Group B	Normal rats	3	3.99 μg	7
		3	8.19	11
		2	6.15	14
Group C	Hypophysectomized rats	3	1.98 μg	11
		1	1.69	12
		2	1.91	13
Group D	Hypophysectomized rats with thyrotropin	3	5.56 μg	11
		3	7.44	12
		4	6.96	13

ing that in the absence of the pituitary thyroid hormone is not discharged, was taken as evidence that in the hypophysectomized rat the thyroid gland binds iodine to protein at a greatly reduced rate. Finally, in the third group of rats it was found that the administration of thyrotropin to hypophysectomized rats previously treated with propylthiouracil restored the ability to accumulate protein-bound iodine at a normal rate. These data are presented in Figure 6 and in Table 2.

5. *The influence of the hypophysis on the discharge of thyroid hormone.*

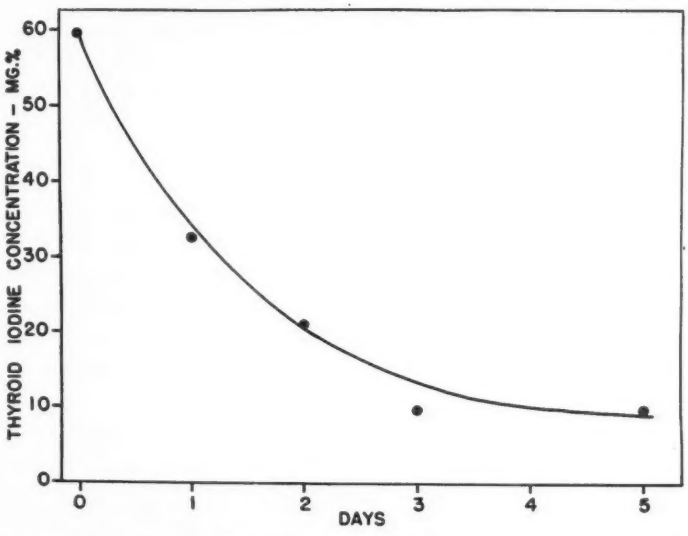

FIG. 7. The discharge of iodine from the thyroid glands of hypophysectomized rats fed propylthiouracil and injected daily with 5 mg. thyrotropin.

This experiment confirmed the observation of Astwood and Bissell (1944) which showed that no decrease in the iodine content of the thyroid gland occurred following the administration of antithyroid drugs to hypophysectomized rats. In addition, it was found that thyrotropin in the doses used caused no decrease in iodine content of the thyroid glands of hypophysectomized rats. However, when hypophysectomized rats were fed a diet containing 0.1 per cent propylthiouracil, the injection of 5 mg. thyrotropin daily was followed by a rapid decline in the iodine content of the thyroid gland, as shown in Figure 7 and Table 3. Further, under these conditions, when thyrotropin was injected in graded doses over a five-day period, the decline in iodine concentration of the thyroid gland bore a direct relationship to the dose used. The data obtained are presented in Figure 8 and

TABLE 3. THE RELATIONSHIP OF THE THYROID IODINE CONCENTRATION IN HYPOPHYSECTOMIZED RATS FED PROPYLTHIOURACIL TO THE HUMBER OF DAILY INJECTIONS OF 5 MILLIGRAMS THYROTROPIN

No. rats	Propylthiouracil	No. daily injections 5 mg. thyrotropin	I_2 mg./100 gm. thyroid
5	+	0	60.3
3	0	5	59.0
4	+	1	32.5
2	+	2	21.2
3	+	3	8.7
5	+	5	9.1

Table 4, and they suggest that the fall in thyroid iodine concentration when thyrotropin is injected into hypophysectomized rats fed propylthiouracil is sufficiently correlated with the dose of thyrotropin so that this procedure could provide an assay method for thyrotropin.

DISCUSSION

The experiments on the control of the hypophysis over the iodide ion concentration of the thyroid gland indicate a marked dependence of this function on pituitary thyrotropin, and in the absence of the pituitary this capacity is almost completely lost at a time when histological changes are barely perceptible.

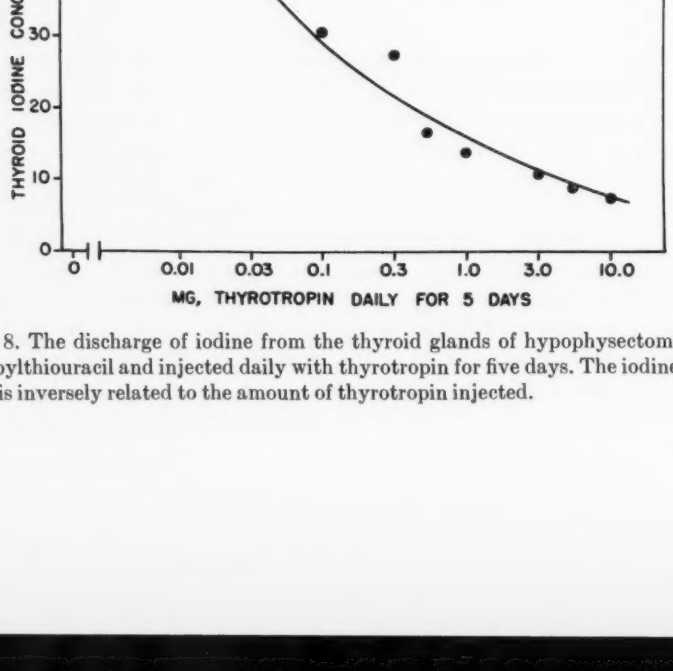

FIG. 8. The discharge of iodine from the thyroid glands of hypophysectomized rats fed propylthiouracil and injected daily with thyrotropin for five days. The iodine concentration is inversely related to the amount of thyrotropin injected.

Since the iodide-concentrating power of the thyroid seems not to depend upon the cellular volume of the gland, the enzyme system responsible for the iodide concentration must be extremely sensitive to a lack of thyrotropin, as indicated by the precipitous fall in thyroid/serum iodide ratio following hypophysectomy, although the cellular/non-cellular ratio of the gland remained relatively constant. The iodide-concentrating mechanism appears to be so regulated that under conditions of varying iodine supply the efficiency of iodide accumulation can be greatly altered; for example, when iodine is deficient, there is a compensatory alteration of thyroid function so that the available supply of iodine can be trapped far more efficiently and thus the need for thyroid hormone can be fully met despite

TABLE 4. THE RELATIONSHIP OF THE THYROID IODINE CONCENTRATION IN HYPOPHYSECTOMIZED RATS FED PROPYLTHIOURACIL TO THE AMOUNT OF THYROTROPIN INJECTED DAILY FOR FIVE DAYS

No. rats	Dose/day thyrotropin mg.	I_2 mg./100 gm. thyroid
5	0	60.3
2	0.01	52.0
3	0.03	41.4
4	0.10	30.3
7	0.3	27.2
3	0.5	16.9
4	1.0	13.0
2	3.0	10.8
5	5.0	9.1
1	10.0	7.6

the relative iodine lack. Although hyperplasia of the thyroid gland ordinarily attends an increased efficiency for accumulating iodine, and hypoplasia, a decreased efficiency, the results recorded here indicate that the function of concentrating iodide ion can be divorced from the anatomical state, for after hypophysectomy thyroid glands which were still hyperplastic from propylthiouracil administration possessed a very low capacity for iodide accumulation.

The data on the binding of iodine to protein indicate that in the rat the thyroid gland retains little ability to form thyroid hormone in the absence of the pituitary. This ability is restored to normal by the daily subcutaneous injection of thyrotropin.

The observations on the influence of hypophysectomy plus injection of thyrotropin, of hypophysectomy plus propylthiouracil-feeding, and of hypophysectomy plus propylthiouracil-feeding plus injection of thyrotropin, indicate that in the doses used neither thyrotropin alone nor propylthiouracil alone causes a decrease in the iodine content of the rat thyroid gland. It appears that the thyroid gland of the hypophysectomized rat receiving thyrotropin functions in a nearly normal fashion and is not depleted of iodine. Propylthiouracil exerts no influence on the iodine con-

tent of the thyroid gland of the hypophysectomized rat, apparently because little or no thyroid hormone is formed or released in the absence of the pituitary. However, the administration of both propylthiouracil and thyrotropin to hypophysectomized rats results in a fall in thyroidal iodine content and concentration which can be related to the number of daily injections of a constant dose of thyrotropin and to the dose used when the period of time is kept constant. A reasonable interpretation of these results is that the pituitary governs the release of thyroid hormone through its thyrotropic hormone and that the amount of thyroid hormone discharged derives directly from the amount of thyrotropin elaborated.

A discrepancy between the data on the iodine content of the thyroid gland of the hypophysectomized rat after large doses of potassium iodide and the data on the thyroid : serum iodide ratio after hypophysectomy is apparent. This has previously been encountered by Morton *et al.* (1942) in comparing their data with those of Leblond and Süe (1941). It would appear that after hypophysectomy slight iodide-concentrating ability persists and perhaps the slow loss of iodide from the thyroid gland after the peak which follows a large dose of potassium iodide is due to diminished excretion of iodide by the kidney.

Morton *et al.* (1942) interpreted their data as indicating that the conversion of iodide to diiodotyrosine is not impaired in the hypophysectomized rat but that thyroxine formation is largely inhibited. They noted that their data were not quantitative, however, and in consideration of the data presented here it would appear that in the hypophysectomized rat binding of iodide to protein occurs at a greatly diminished rate and in view of their data that practically no thyroxine formation occurs. This interpretation is hard to reconcile with the finding of Taurog, Chaikoff and Bennett (1946) that after hypophysectomy the protein-bound plasma iodine level of the rat declines only to about 50 per cent of the normal value and raises some question of the significance of that determination in this species.

CONCLUSIONS

Under the conditions of the experiments reported it appears that the thyroid gland of the rat is dependent on the presence of thyrotropic hormone for its capacity to concentrate iodide from the serum, and following hypophysectomy this capacity is lost to a far greater extent than can be accounted for on the basis of anatomical changes in the thyroid gland.

Data are presented which indicate that the thyroid gland of the hypophysectomized rat binds little iodine to protein; this function is restored when thyrotropin is injected subcutaneously.

This study indicates that discharge of thyroid-hormone depends on the

pituitary thyrotropin and does not occur in hypophysectomized rats. When hypophysectomized rats are treated with propylthiouracil to inhibit further formation of thyroid hormone, the injection of thyrotropin results in a fall in thyroid iodine concentration which is directly related to the amount of thyrotropin injected.

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THE EFFECT OF HYPOPHYSECTOMY, ADRENALECTOMY AND OF ACTH ADMINISTRATION ON THE PHOSPHORUS METABOLISM OF RATS[1]

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SINCE phosphorus metabolism is intimately linked with both electrolyte and energy exchanges, it is logical to expect that the pituitary and adrenal gland hormones would affect it. Yet the literature indicates considerable confusion regarding such influence. This is due in part to failure to compare the effects on different organs and in part to an inability to determine the changes in movement, which account for the shifts in concentration.

Following hypophysectomy according to Anderson and Oestler (1948, cats), Jones and Shinowara (1942, dogs) and Li, Geschwind and Evans (1949, rats) the serum inorganic phosphorus decreases. The only purified anterior pituitary hormone which has been demonstrated to influence the serum inorganic phosphorus is the growth hormone. Li, Geschwind and Evans (1949) have shown in rats that this hormone prevents the fall of inorganic phosphorus in serum after hypophysectomy and even elevates the phosphorus level above that of the controls.

The effect of ACTH on the inorganic phosphorus of the plasma has been reported with varying and conflicting data. Clinical data from Forsham, Thorn, *et al.* (1948) show a decrease in serum inorganic phosphate after administration of a large dose of ACTH to a patient with pituitary insufficiency and another subject with asthenia, but an increased serum inorganic phosphate after injection of the hormone in a normal subject. The changes in serum inorganic phosphorus in these individuals were associated with changes in urinary phosphorus excretion; there was an increased phosphorus excretion in the two first subjects and a decrease in the normal one. Later, however, Mason, *et al.* (1949) and Sayers, *et al.* (1949) failed to find any differences in serum inorganic phosphorus or urinary

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excretion after administration of single or multiple injections of ACTH into three healthy persons.

Tornblom (1949) administered ACTH to hypophysectomized rabbits which were kept on a phosphate-deficient diet rich in calcium. He found that the serum inorganic phosphorus which was decreased after hypophysectomy was increased by administration of the hormone.

In the present studies, P^{32} has been used to measure exchange, and the changes in both amount and turnover have been determined in a number of major tissues. An attempt has been made to study the effect of hypophysectomy, adrenalectomy and ACTH injection on phosphate metabolism.

METHODS

Male and female rats of the Wistar strain were used in these studies. Hypophysectomy was performed at about 40 days of age by the parapharyngeal approach and the phosphorus experiments were carried out 5–6 days later. The completeness of the removal of the gland was ascertained at autopsy by the examination of the sella turcica. Adrenalectomy was performed at about 40 days of age by the usual method. The animals were used 24 hours after the operation. During the interval between operation and experiment all rats were maintained on the usual diet of this laboratory *ad libitum*. They were fasted 12 hours before the experiment.

The injections of the adrenocorticotrophic hormone (ACTH)[3] were given intraperitoneally in isotonic NaCl solution 6 hours before killing the animal. At the time of the experiment 0.04 millicurie of P^{32} was injected intraperitoneally in 1.0 ml. of physiological saline solution, irrespective of the weight of the animal. The total phosphate given never exceeded 0.1 mg., a physiologically negligible amount.

The animals were anesthetized with ether and blood was taken from the abdominal aorta with heparin as the anticoagulant. The blood was immediately centrifuged and the plasma separated. Portions of the brain, liver, kidney, muscle, and intestine and both adrenals were taken and dropped in dry ice as soon as possible. From each organ we have always tried to take the same part as exactly as possible. A slice of about 100 mg. from the left anterior lobe of the cerebrum was chosen. From the lower pole of the left kidney a piece of similar weight was taken, the kidney pelvis being avoided. The muscle piece was taken from the quadriceps muscle of the left hind leg. A section of the small intestine 1 cm. long was removed just below the duodenum. Both the adrenals were combined in the same sample.

For estimation of plasma phosphate, 0.3 ml. of the plasma was pipetted into 2 ml. cold 10% trichloracetic acid and filtered quantitatively into a 10 ml. volumetric flask. The filter was washed with the trichloracetic acid reagent and the contents of the flask were made up to volume with water. From this extract aliquot parts were taken for phosphate and radioactivity determinations.

The tissues were transferred to a tube containing 2.0 ml. cold 10% trichloracetic acid and ground with quartz sand. After quantitative filtration into a 10 ml. volumetric flask aliquot parts were taken for measurement of phosphate and radioactivity.

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[3] We wish to thank Dr. C. W. Mote and Dr. Edwin M. Hays of Armour and Company for the supplies of hormone.

For the determination of phosphate the Fiske-Subbarow method as modified by Briggs (1922) was employed. The determinations on plasma were always done within 2 hours and those on the tissues were done the same day. The radioactivity in these samples was determined according to a method worked out at the Wenner-Grens Institut in Stockholm by Levi (1941).

DEFINITIONS

ACTIVITY in a sample denotes the presence of labeled material. Its magnitude is indicated by the intensity of β-ray radiation and is expressed as the number of counts per minute per 0.001 ml. plasma or per mg. tissue.
SPECIFIC ACTIVITY—Number of counts per 0.001 mg. phosphorus.
RELATIVE SPECIFIC ACTIVITY is the specific activity of tissue compared to the specific activity of plasma.
RELATIVE ACTIVITY is the number of counts per mg. tissue compared to the activity in plasma.

RESULTS

Blood

Since a constant dose of P^{32} was injected into each rat independent of size, it was necessary when blood samples from different animals were to be compared to correct the activity for varying dilution. For a constant general composition, the dilution would be proportionate to the weight. The activity found in a certain volume of plasma, therefore, was multiplied by the weight of the animal to give a value (corrected activity) independent of the size of the animal (Table 1).

TABLE 1. THE AMOUNT AND SPECIFIC ACTIVITY OF INORGANIC PHOSPHORUS IN PLASMA 50 MINUTES AFTER AN INTRAPERITONEAL INJECTION OF P^{32}

Group	No. of rats	Body weight A	Inorganic phosphorus B	Activity C	Corrected activity AC	Specific activity C/B	Corrected spec. activity AC/B
		gm.	mg. per 100 ml.	cts. per μl.	gm. cts. per μl.	cts./mg. $\times 10^{-3}$	gm. cts. per mg. P.
Normal	17	155 ± 3.1	5.36 ± 0.18	17.00	2,640 ± 126	317	493
Normal-ACTH	5	164 ± 2.9	4.55 ± 0.15*	13.40	2,210 ± 198	295	485
Adrenalectomized	8	179 ± 10.7	5.04 ± 0.17	14.60	2,480 ± 173	290	493
Hypophysectomized	12	128 ± 3.7*	4.45 ± 0.17*	28.00	3,510 ± 164*	630*	790*
Hypophysectomized-ACTH	12	130 ± 5.1*	3.95 ± 0.20*	23.00	2,810 ± 153	558*	712*

* Difference from normal >3 ×standard error of the mean.

The curves in Figure 1 represent the corrected specific activity of plasma in normal and hypophysectomized rats 20, 40, 120 minutes and 24 hours after a single intraperitoneal injection of P^{32}. Throughout the first 24 hours the curve for the hypophysectomized rats is higher than the curve for the normal animals and the relationship between the two curves remains about the same. The fact that the difference was marked as early as 20 minutes after injection and remained fairly constant throughout the period, would indicate that this was not primarily due to differences in rate of absorption or in rate of turnover between the different body compart-

ments. It would appear that the radiophosphate was diluted differently in the two types of animals. Since plasma phosphate rapidly comes into equilibrium with the inorganic phosphate of the interstitial space but only slowly with the intracellular compartment (Hevesy, 1939), it would appear that hypophysectomy reduced the extracellular space in relation to the total volume of the animal.

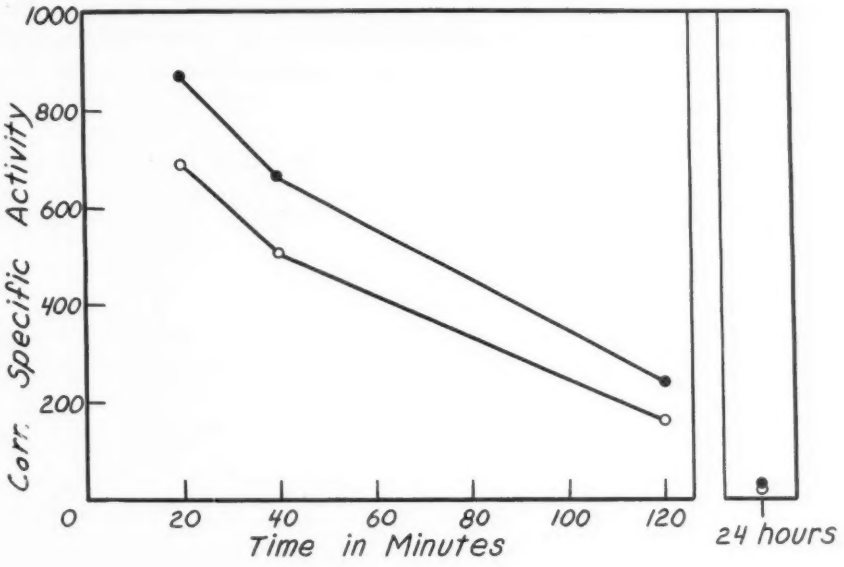

FIG. 1

During the five days between hypophysectomy and the injection of the isotope, the hypophysectomized animals lost an average of 20 grams, while the controls gained 7 grams per animal. If we assume that the total difference of 27 grams per animal was due to loss of water from the hypophysectomized rats, an obvious overcorrection, the normal animals would have a corrected specific activity of 18.50 counts per mg. plasma while the same value for the hypophysectomized rats would be 23.00 counts per mg. plasma. This is still a highly significant difference between the two groups. It would seem then, that while a major factor in the difference in specific activities was a reduced extracellular space in the hypophysectomized animals, there was, in addition, a reduced rate of exchange between the intracellular and extracellular compartments.

In spite of the reduced extracellular space associated with hypophysectomy, there was a significant reduction in the absolute level of inorganic phosphate. The loss of ion from the extracellular fluid, therefore, was

greater than the loss of water. This could either have been more rapid excretion, deposition in bone, or greater transfer to intracellular space following the operation. The latter seems unlikely since there were no significant changes in concentration of tissue phosphate.

When adrenalectomy was performed 24 hours before the experiment, there was no significant change in either the inorganic phosphate level of the plasma or the corrected specific activity. When ACTH was administered to either normal or hypophysectomized rats however, there was a significant decrease in the concentration of inorganic phosphate but no significant effect on the corrected specific activity of the blood. This is the type of change which might be expected if there was a simple dilution of the phosphate in the extracellular space. Since there was no significant increase in the concentration of inorganic phosphate in any of the tissues it is highly probable that the dilution was achieved by reduced excretion of water.

Liver

The only significant change in the concentration of inorganic phosphate in the liver was an increase in the adrenalectomized animals (Table 2).

TABLE 2. THE AMOUNT AND RATE OF TURNOVER OF INORGANIC PHOSPHORUS IN LIVER 50 MINUTES AFTER AN INJECTION OF P^{32}

Group	No. of rats	Plasma		Liver				
		Activity A	Specific activity B	Inorganic phosphorus C	Activity D	Specific activity D/C	Relative spec. act. D/BC	Relative activity D/A
		counts/mg. (μl.) plasma	cts./mg. P. $\times 10^{-3}$	mg./100 gm. liver	cts./1 mg. liver	cts./mg. $\times 10^{-3}$	$\times 10^{2}$	
Normal	17	17.00	317	28.9±2.2	47.0±4.4	162	51.3±2.1	2.77
Normal-ACTH	5	13.40	295	26.2±1.4	44.7±2.7	171	57.8±3.6	3.33
Adrenalectomized	8	14.60	290	34.5±1.1	65.8±3.4	191	65.7±1.2	4.51
Hypophysectomized	12	28.00*	630*	28.4±2.4	63.8±5.8	224	35.6±4.1*	2.28
Hypophysectomized-ACTH	12	22.00*	558*	30.8±2.1	92.0±4.8*	299	53.5±2.9*	4.19

* Difference from normal >3 ×standard error of the mean.

This was not associated with any change in the inorganic phosphate of the plasma, and therefore must have represented a change in the intracellular concentration of inorganic phosphate. This could have arisen through an increased breakdown of phosphate esters in the cell, by loss of water from the cell, or by a shift of phosphate ions into the cell. In the first case, the excess ions would have originated from sources which would have had a low concentration of isotope and the relative specific activity would therefore have been less than in the normal animals. Since the opposite was the case, it must be assumed that one of the other two changes must have been responsible for the increased concentrations, and also that the process was not at equilibrium, but that the change was going on during the presence of the isotope.

In the case of the hypophysectomized animals, the low relative specific activity would indicate a decreased turnover of inorganic phosphate between the intracellular and extracellular phases, compared with the turnover between intracellular inorganic phosphate and ester phosphate. Since the reduced metabolic rate of the hypophysectomized animal would indicate that the latter process was diminished rather than increased, it seems highly probable that the low relative specific activity represents a decreased turnover between extracellular and intracellular phosphate. If the decreased turnover was due solely to the lower concentration of phosphate ions in the extracellular fluid, the relative activities should

TABLE 3. THE AMOUNT AND RATE OF TURNOVER OF INORGANIC PHOSPHORUS IN KIDNEY 50 MINUTES AFTER AN INJECTION OF P^{32}

Group	No. of rats	Plasma		Kidney				
		Activity A	Specific activity B	Inorganic phosphorus C	Activity D	Specific activity D/C	Relative spec. act. D/BC	Relative activity D/A
		counts/mg. (μl.) plasma	cts./mg. P. $\times 10^{-2}$	mg./100 gm. kidney	cts./100 mg. kidney	cts./mg. $\times 10^{-3}$	$\times 10^{2}$	
Normal	17	17.00	317	27.1±1.1	55.5±2.4	205	64.7±2.1	3.27
Normal-ACTH	5	13.40	295	28.1±1.2	53.5±4.1	190	64.6±4.3	3.99
Adrenalectomized	8	14.60	290	31.1±2.2	57.1±3.6	184	63.3±2.7	3.92
Hypophysectomized	12	28.00	630	32.4±1.6	91.0±5.1	281	44.6±3.9*	3.25
Hypophysectomized-ACTH	12	22.00	558	29.1±1.6	100.0±7.1	344	61.8±3.2	4.54

* Difference from normal >3 ×standard error of the mean.

be the same as normal. Since this is also decreased, we must assume a decrease in the actual rate of transfer per mole of ion.

When ACTH was administered to the hypophysectomized rats, the relative specific activity of the liver inorganic phosphate was restored to normal levels. In part, this was due to the lower specific activity of the plasma due to water retention. There also must have been an actual increase in the transfer of phosphate between extracellular and intracellular space compared with the rate of transfer into phosphate esters. This would appear to have been raised significantly above normal. Since adrenalectomized animals showed a similar increase in relative activity, the effect of ACTH on this process cannot be attributed to its action on the adrenals. Apparently, some other mechanism was involved. Whether this was the effect of some contaminating hormone cannot be answered at this time.

Kidneys

Hypophysectomized animals showed a decreased relative specific activity of the kidney inorganic phosphate (Table 3). Unlike the liver, however, this appeared to be due to the dilution of phosphorus in the extracellular fluid, since the relative activity did not differ from that of the normal animals. Again, the injection of ACTH into the hypophysectomized ani-

mals restored the relative specific activity to normal even though the extracellular phosphate was further diluted. The relative activity was raised significantly above normal, again indicating a relatively rapid transfer of the ion across the cell interface as compared with its incorporation into phosphate esters. As in the case of the liver, this was not a reversal of the findings in the adrenalectomized animal where no significant variation from normal was observed. It must therefore either be an effect of the pituitary extract which is not mediated through the normal function of the adrenal, or one which requires so little hormone that the circulating levels of the latter were not reduced to a critical level by removal of the glands 24 hours previously.

Adrenal

In the adrenal gland (Table 4) hypophysectomy, as we would expect, had a very marked effect. The concentration of inorganic phosphorus in

TABLE 4. THE AMOUNT AND RATE OF TURNOVER OF INORGANIC PHOSPHORUS IN ADRENAL GLAND 50 MINUTES AFTER AN INJECTION OF P^{32}

Group	No. of rats	Plasma		Adrenal gland				
		Activity A	Specific activity B	Inorganic phosphorus C	Activity D	Specific activity D/C	Relative spec. act. D/BC	Relative activity D/A
		counts/mg. (μl.) plasma	cts./mg. P. $\times 10^{-3}$	mg./100 gm. adrenal	cts./100 mg. adrenal	cts./mg. $\times 10^{-3}$	$\times 10^{2}$	
Normal	17	17.00	317	26.6±1.6	18.4±1.4	69.2	21.8±1.9	1.08
Normal-ACTH	5	13.40	295	28.7±2.3	27.2±5.1	94.8	32.1±2.1	2.03
Hypophysectomized	12	28.00	630	34.3±2.5	19.4±1.2	56.6	9.0±0.9*	0.69
Hypophysectomized-ACTH	12	22.00	558	28.1±2.1	35.2±4.6	125.3	22.5±2.0*	1.60

* Difference from normal >3 ×standard error of the mean.

the glands increased significantly. The turnover rate of this inorganic phosphorus was decreased more than 50% indicating the reduced metabolic activity of the cells. After administration of ACTH there was an increased relative specific activity and relative activity in both normal and hypophysectomized animals due to the more rapid interchange of ions across the surface of the active cells. In the hypophysectomized animals the concentration of inorganic phosphorus was also decreased to normal.

Intestine

Here again, the reduced relative specific activity and relative activity indicated a slower turnover of phosphate between cells and surrounding fluid in the hypophysectomized rats (Table 5). Again the injection of ACTH restored the relative specific activity to normal. The relative activity was also raised to normal but not significantly increased above this level as in the case of the kidney and liver. Again, this effect upon the

TABLE 5. THE AMOUNT AND RATE OF TURNOVER OF INORGANIC PHOSPHORUS IN INTESTINE 50 MINUTES AFTER AN INJECTION OF P^{32}

Group	No. of rats	Plasma		Intestine				
		Activity A	Specific activity B	Inorganic phosphorus C	Activity D	Specific activity D/C	Relative spec. act. D/BC	Relative activity D/A
		counts/mg. (μl.) plasma	cts./mg. P. $\times 10^{-3}$	mg./100 gm. intestine	cts./100 mg. intestine	cts./mg. $\times 10^{-3}$	$\times 10^{2}$	
Normal	17	17.00	317	36.6±1.7	39.5±2.1	108	34.1±1.3	2.32
Normal-ACTH	5	13.40	295	33.7±2.1	37.6±2.1	111	37.8±3.0	2.81
Adrenalectomized	8	14.00	290	36.7±1.9	39.3±1.8	107	36.9±2.4	2.59
Hypophysectomized	12	28.00	630	33.6±1.4	48.0±3.3	143	22.7±1.3*	1.72
Hypophysectomized-ACTH	12	22.00	558	29.5±1.4	55.9±2.8	189	34.0±1.4	2.54

* Difference from normal >3 ×standard error of the mean.

decreased rate of exchange in the hypophysectomized animal could not be explained by restoration of normal adrenal function, since adrenalectomy did not lead to the opposite change.

Muscle

As observed by others (Hevesy, 1939), the exchange of inorganic phosphate between the extracellular fluids and the muscle fiber is much slower than in such tissues as the liver, kidney and intestine. This was the only tissue investigated in which adrenalectomy 24 hours earlier reduced the

TABLE 6. THE AMOUNT AND RATE OF TURNOVER OF INORGANIC PHOSPHORUS IN MUSCLE 50 MINUTES AFTER AN INJECTION OF P^{32}

Group	No. of rats	Plasma		Muscle				
		Activity A	Specific activity B	Inorganic phosphorus C	Activity D	Specific activity D/C	Relative spec. act. D/BC	Relative activity D/A
		counts/mg. (μl.) plasma	cts./mg. P. $\times 10^{-3}$	mg./100 gm. muscle	cts./100 mg. muscle	cts./mg. $\times 10^{-3}$	$\times 10^{2}$	
Normal	17	17.00	317	66.5±2.8	5.48±0.26	8.2	2.60±0.20	0.323
Normal-ACTH	5	13.40	295	63.1±3.7	5.51±0.61	8.7	2.96±0.41	0.412
Adrenalectomized	8	14.60	290	66.8±3.0	3.45±0.25	5.2	1.78±0.14*	0.235
Hypophysectomized	12	28.00	630	62.0±3.5	4.53±0.23	7.4	1.16±0.11*	0.162
Hypophysectomized-ACTH	12	22.00	558	59.5±5.3	4.90±0.35	8.2	1.48±0.11	0.223

* Difference from normal >3 ×standard error of the mean.

rate of turnover (Table 6). Hypophysectomy produced an even more marked reduction in the exchange between the muscle cell and its environment. Unlike the other tissues already discussed, the dose of ACTH used in these experiments, while it increased the rate of turnover in the muscle of the hypophysectomized animal, did not restore it to normal. Whether this was because the extract did not contain some other factor essential for normal phosphate metabolism in muscle tissue, or whether the muscle required a larger amount of the cortical hormones was not determined. Certainly in the muscle of the hypophysectomized animal, it would appear that at least two processes were interfered with: that which affected the cells of the other tissues as well, and that due to reduced adrenal function

which seemed to have relatively little influence on phosphorus metabolism in other tissues in this length of time.

Brain

Brain (Table 7) showed even a slower exchange of phosphate between cells and interstitial fluid (Hevesy and Ottesen 1943). Adrenalectomy 24 hours earlier had no effect on this tissue. Hypophysectomy, however, again caused a reduced relative specific activity and relative activity, indicating again an effect on exchange between intracellular and extracellular phos-

TABLE 7. THE AMOUNT AND RATE OF TURNOVER OF INORGANIC PHOSPHORUS IN BRAIN 50 MINUTES AFTER AN INJECTION OF P^{32}

Group	No. of rats	Plasma		Brain				
		Activity A	Specific activity B	Inorganic phosphorus C	Activity D	Specific activity D/C	Relative spec. act. D/BC	Relative activity D/A
		counts/mg. (μl.) plasma	cts./mg. P. $\times 10^{-3}$	mg./100 gm. brain	cts./100 mg. brain	cts./mg. $\times 10^{-3}$	$\times 10^{2}$	
Normal	17	17.00	317	33.5±1.3	1.50±0.09	4.5	1.41±0.13	0.088
Normal-ACTH	5	13.40	295	32.1±2.3	1.47±0.10	4.6	1.55±0.22	0.110
Adrenalectomized	8	14.60	290	35.4±2.0	1.47±0.11	4.2	1.43±0.11	0.105
Hypophysectomized	12	28.00	630	33.8±0.9	1.65±0.11	4.9	0.78±0.07*	0.059
Hypophysectomized-ACTH	12	22.00	558	33.5±1.8	2.13±0.16	6.4	1.14±0.08*	0.097

* Difference from normal >3 ×standard error of the mean.

phate which was not due to the reduced concentration in the extracellular spaces. The injection of ACTH again increased the rate of turnover of cellular phosphate over and above any influence of the extracellular concentration. As in the case of muscle, however, the restoration did not appear to be complete. It may be that the reduced effect of the extract in muscle and brain was due to slower penetration of these cells by the active component in the blood.

In summary, it would appear that hypophysectomy had an effect on the phosphorus metabolism of all tissues which was not duplicated by an absence of adrenal secretion for 24 hours. Some factor in the adrenocorticotrophic extract which was used would offset this decreased phosphate exchange. The phosphate metabolism of muscle was also reduced by the removal of the adrenals, and this, in addition to the other effect, made the influence of hypophysectomy more marked than on any other tissue except the adrenals. The adrenals were, as might have been expected, most markedly affected by hypophysectomy, and were greatly stimulated by ACTH in both the hypophysectomized and normal animals.

DISCUSSION

The effect of hypophysectomy on the plasma inorganic phosphorus is already well known. The present work, however, shows that the decreased concentration is not due to a dilution, in fact the extracellular space in

which the inorganic ion is distributed is reduced. Neither is the reduction due to storage in any of the major soft tissues. It must, therefore, either be due to an increased excretion or to increased storage in the bones. The reduction in extracellular phosphate must be considerable since there is reduced concentration in a reduced space.

It was pointed out that after removal of the hypophysis there was also a decrease in the relative specific activities of the inorganic phosphate of all the tissues measured. Since the measurements were made before equilibrium had been established, this could mean either that the inorganic ion in the cell was exchanging more rapidly with the phosphate esters of the cell or that the rate of exchange between the extracellular phosphate and that in the cell was decreased. The ester phosphate was not measured but, since the metabolic rate of the hypophysectomized rat is decreased, it is highly improbable that the turnover of phosphate esters was increased. It seems highly probable, therefore, that there was a general decrease in the transfer of phosphate across cell boundaries, a process which seems to involve energy.

This process of transfer of phosphate seems to be under the control of some factor contained in the adrenocorticotrophic extract which was used. In every tissue studied the decreased turnover of the hypophysectomized rats was increased on administration of one dose of ACTH; in all except muscle and brain to that of the normal controls. Since removal of the adrenals 24 hours previously did not produce a significant fall in the relative specific activity of any tissue except muscle, there seem to be only two possible explanations for the effect of the extract: either the level of the adrenal compound involved did not fall below effective levels in this length of time, or that the extract had an effect which was not mediated through the adrenal glands. In the latter case it would not appear to be due to the other known hormones of the hypophysis since, according to the research laboratories of Armour and Company, this material had only traces of any other activity. The phosphate effect should be checked with several highly purified preparations.

It is perhaps not surprising that muscle should show the most rapid and complicated response to the removal of the adrenal glands. The turnover of phosphate in muscle is rapid and the dependence of the contractile process on the adrenal steroids has been emphasized by the work of Ingle (1944) and Ingle and Nezamis (1948). If, as Sayers and Sayers (1949) think the adrenal steroids are metabolized by the tissues in which they exert their effects, one would expect that the evidence of their absence in reduced phosphate turnover might first be seen in muscle tissue.

Since, in addition to the marked role of the adrenal cortex in contracting

muscle, the thyroid and growth hormones also affect its metabolism, the pattern of disturbance in the hypophysectomized animal might be expected to be more complicated. ACTH alone could hardly be expected to restore a normal rate of turnover.

The adrenal cortex itself would, of course, be rapidly affected by hypophysectomy. After this operation the retrogression of the adrenal gland is so rapid that the size of the cortex may decrease one-half in six days (Smith 1927, 1930). The increase in concentration of inorganic phosphate during this time probably is due to decreased incorporation in esters and phospholipids. The exchange of phosphorus in phospholipids and nucleotides of the adrenal gland is very small and, therefore, could not be responsible for the large change in turnover rate. Forty minutes after an injection of P^{32} the turnover rate of phosphorus in these two compounds is only 0.5% of that of inorganic phosphorus in the same gland (Gemzell 1948). According to Gemzell (1948) an increased turnover rate of acid soluble organic phosphorus in the adrenal gland of a normal rat is followed by an increased secretion of adrenal steroids, which was demonstrated by an increased liver glycogen content in fasting animals. The variations in relative specific activity therefore, are probably a reflection of changes in synthesis and secretion of hormones, and indicate that the amounts of ACTH used set free significant amounts of adrenal hormones in both the hypophysectomized and normal rats.

In spite of the marked effect of ACTH on the exchange of phosphate between tissues and circulating fluids, it is interesting that the plasma phosphate levels, instead of being restored toward normal, were still further lowered. On the other hand, it has been shown by Li, *et al.* (1949) that growth hormone increases the plasma phosphate level. Since the levels of phosphate ion in the tissues were not significantly affected by hypophysectomy it would appear that the growth hormone must supply the additional phosphate through affecting kidney function, by its influence on bone, or by decreasing the amount of phosphorus bound as ester.

While the work presented does not answer many questions regarding the relation of the hypophysis to phosphorus metabolism, it does demonstrate the marked influence of adrenocorticotrophic hormone on the regulation of the energy systems involved in the maintenance of the normal relations between the extracellular and intracellular environment. It also raises the question of a possible action of this hormone by a route other than the adrenal.

SUMMARY

The turnover of inorganic phosphate between plasma and tissues in normal rats has been compared with that of animals which were adrenalec-

tomized, hypophysectomized, or hypophysectomized and treated with adrenocorticotrophic hormone, using P^{32} as a tracer. Hypophysectomy markedly reduced the rate of turnover of inorganic phosphate between the cells and the extracellular fluids of all the tissues measured. It did not significantly affect the concentration in these tissues, however, even though the plasma level was reduced by 18%. Adrenalectomy 24 hours before the injection of the isotope did not significantly affect the turnover of phosphorus in any tissue except the muscle.

The injection of adrenocorticotrophic hormone into hypophysectomized rats increased the rate of turnover in all of the tissues studied. It restored the rate to normal in all tissues except muscle and brain. Plasma phosphate was further reduced, however, apparently due to retention of water. Apparently the systems involved in the transfer of phosphate across cell boundaries are under the control of ACTH; whether this is direct or via the adrenals is not clear. The regulation of the plasma phosphate level, however, depends on some other pituitary factor, probably the growth hormone.

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PRODUCTION OF REVERSIBLE HYPERADRENOCORTINISM IN RATS BY PROLONGED ADMINISTRATION OF CORTISONE

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WITH the use of cortisone in the treatment of rheumatoid arthritis and allied conditions (Hench, Kendall, Slocumb and Polley, 1949; Hench, Slocumb, Barnes, Smith, Polley and Kendall, 1949), signs of hyperadrenocortinism may be expected in at least some cases treated over a prolonged period of time. Reactions consisting of one or more of the manifestations of Cushing's syndrome have been observed in a small percentage of patients treated with cortisone (Sprague, 1949). It was therefore of interest to determine in animals what signs of hyperadrenocortinism could be produced by daily administration of large doses of cortisone, and particularly whether the effects observed were reversible after cessation of treatment with the hormone.

MATERIALS AND METHODS

The animals used in this study were male Holtzman rats, all of the same shipment and weighing between 180 and 210 gm. The animals were housed in individual cages and fed a stock diet, with water *ad libitum*. The cortisone acetate employed was CORTONE (Merck), which is a saline suspension of the micronized steroid. In these experiments, the concentration used was 5 mg. per ml.[1]

Fifty rats (Group I) received subcutaneous injections of 3 mg. of cortisone acetate daily. Ten of these were sacrificed after 10 injections, and in 40, administration was continued for 6 weeks. Ten of the latter were sacrificed at the end of the injection period, and the remainder at intervals after discontinuance of injections as follows: 4 days after last injection, 4 rats; 10 days, 10 rats; 17 days, 4 rats; 45 days, 12 rats.

In 10 control rats (Group II), food intake was restricted so that their average body weight was approximately the same as that of the cortisone-treated rats. An additional 9 control animals (Group IIIa) received daily injections of 3 mg. of cholesterol in a suspending medium of the same composition as that used for cortisone. This control suspension was similar to that used by Hench and collaborators as placebo medication in human patients. Thirty untreated controls constituted Group IIIb. The rats of Group III were sacrificed at various times, to provide appropriate control data for the animals of Group I.

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[1] We are indebted to T. J. Macek and P. J. Daughenbaugh of the Research Laboratories of Merck & Co., Inc., for the preparation of the suspension. The composition of the suspension is the same as that employed by Hench and his collaborators at the Mayo Clinic in the majority of their patients, except that the suspension for clinical use contains 25 mg. of cortisone acetate per ml.

The animals were weighed at least twice weekly throughout the experiment. Food consumption was measured 4 to 5 days a week in 10 of the injected animals and in 10 of the controls. Animals were sacrificed under pentobarbital anesthesia, and at autopsy, samples of blood were taken for biochemical analysis, organs were weighed, and samples of tissues were fixed in Bouin's solution for histological study. Adrenals were assayed for content of ascorbic acid and cholesterol.

The biochemical methods used were as follows: serum protein fractions, Kibrick and Blonstein (1948); cholesterol, Sheftel (1944); plasma fibrinogen, Cullen and Van Slyke (1920); glucose, Horvath and Knehr (1941); alkaline phosphatase, Bodansky (1933); urea nitrogen, Conway and O'Malley (1942); allantoin, Young (1944); serum calcium, Halverson and Bergeim (1917), without deproteinization; ascorbic acid, Roe and Kuether (1943); serum sodium and potassium, by the Beckman flame photometer.

RESULTS

Body weight. The dose of cortisone used had a profound inhibiting effect on growth, as can be seen in Fig. 1. The treated animals weighed practically

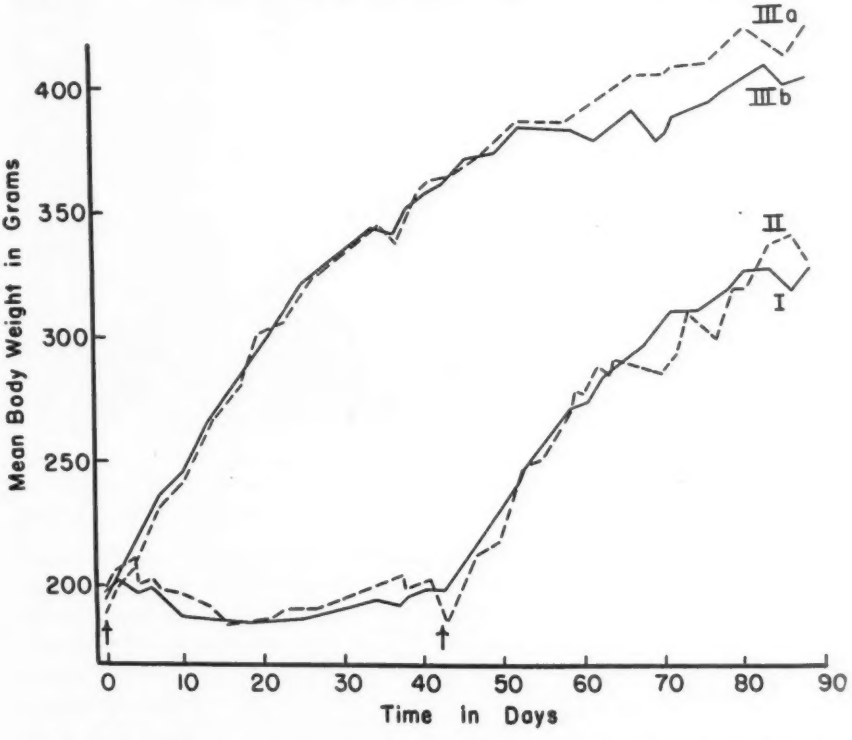

FIG. 1. Growth curves of rats: Group I, 3 mg. of cortisone acetate daily for 6 weeks; arrows indicate beginning and end of injections. Group II, uninjected animals with food intake restricted to simulate body weight changes of cortisone-injected rats. Group IIIa, cholesterol-injected controls. Group IIIb, uninjected controls food *ad libitum*.

the same after 6 weeks of injection as they did at the beginning, while the uninjected and cholesterol-injected controls nearly doubled their weight during the same period. An inhibiting effect of cortisone on growth has been previously observed by Wells and Kendall (1940) and by Ingle (1941).

When the injections were discontinued, the animals started to gain weight rapidly after about a two-day latent period. During the 45 day

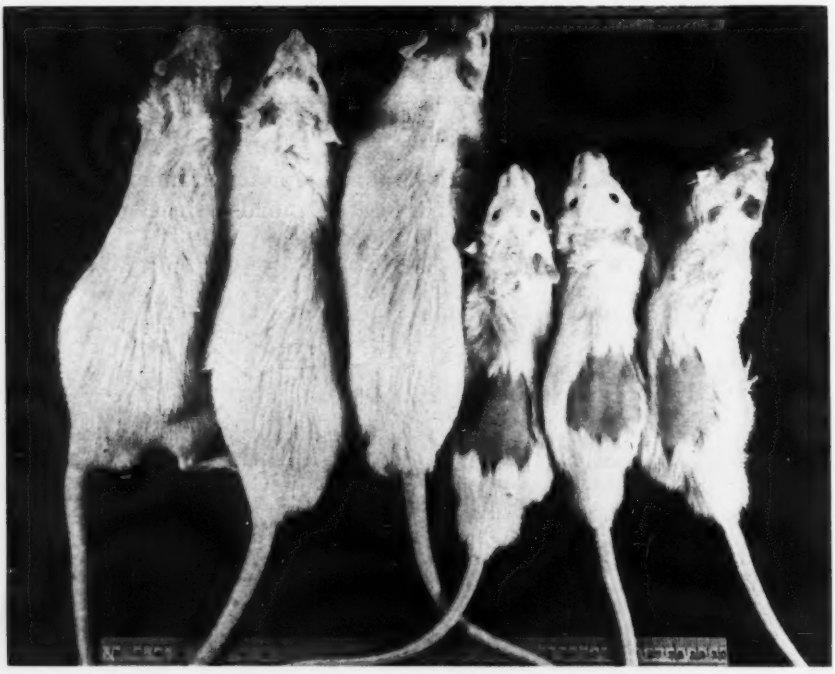

FIG. 2. Effect of cortisone, 3 mg. daily for 6 weeks, on somatic growth and on regrowth of hair. An area on the back was denuded of hair 35 days before this picture was taken. Three animals on left, uninjected controls. To the right, 3 rats on last day of injection.

period of observation after discontinuance of cortisone, the average weight gain of the treated animals was 129 gm. This was about three times the rate of gain of the controls at this time, but was about the same as the growth rate which the controls had shown when they were of a similar weight at the beginning of the experiment. Previous workers have shown (Jackson 1925) that when animals are allowed to eat *ad libitum* after a long period of food restriction, they gain much more rapidly than do normal animals of the same weight. Our rats did not show this excessive rate of weight gain after discontinuance of cortisone.

Food consumption. During the injection period, the cortisone treated animals ate an average of 14.4 gm. of diet daily, compared to 20.3 gm. for the controls. This fact partially accounts for the failure of the injected rats to gain weight normally, but does not explain it fully, for the body weight control animals (Group II) were fed only 9.8 gm. daily on the average, to maintain the same weight as the treated animals. The injected rats therefore ate 47% more food than their body weight controls. This effect proved to be reversible, for during the 45 day post-injection period, the treated animals consumed an average of 18.1 gm. of food daily, compared to an average of 18.5 gm. daily fed to the body weight controls.

Effect on skin and hair. Whitaker and Baker (1948, 1949) have shown that cortisone acetate inhibits hair growth in rats when it is administered by inunction. Furthermore, it is well known that the skin shows atrophic changes in Cushing's syndrome. We have therefore made some studies on skin and the growth of hair in our animals. On ten of the rats injected with cortisone and ten controls, an area on the back about 3 cm. in diameter was denuded by plucking, one week after beginning of the injections. At the end of the injection period, the denuded areas in the treated rats had shown practically no regrowth of hairs, while in the uninjected animals hair regrowth was so complete that the plucked areas were no longer distinguishable. Figure 2, taken on the last day of injection, shows this result, as well as the difference in body size at this time.

Four of the plucked animals were sacrificed at the time the injections were discontinued, and specimens of the skin in the denuded area were fixed in Bouin's, sectioned, and stained with hematoxylin-eosin. Histological examination showed atrophy of the skin and its accessory structures similar to that described by Whitaker and Baker (1949).

The effect of cortisone on hair growth was not permanent, for some regrowth was visible within 4 days after cessation of treatment, and the hair had completely regrown within 3 weeks, or in about the same length of time it had taken for complete regeneration of hair in the uninjected controls.

Adrenal atrophy. Atrophy of the adrenal cortex is a well known consequence of injection of adrenocortical extract (Ingle 1939), cortisone (Wells and Kendall 1940), or desoxycorticosterone (Carnes, Ragan, Ferrebee and O'Neill 1941). Knowlton, Loeb, Seegal and Stoerk (1949) have shown that this effect of desoxycorticosterone is reversible. Since cortisone treatments in human patients may continue for many weeks, it was of interest to determine whether the atrophy produced by such large doses as herein employed in rats would be followed by complete recovery of the gland.

Figure 3 shows that after 10 days' treatment with 3 mg. daily of cortisone acetate, the adrenal glands lost about 40% of their weight, while in 6 weeks the loss amounted to over 50%. Reversal of the atrophy took place rather promptly. Recovery was detectable 4 days after cessation of treatment, was well advanced within 10 days, and was unquestionably complete in 45 days.

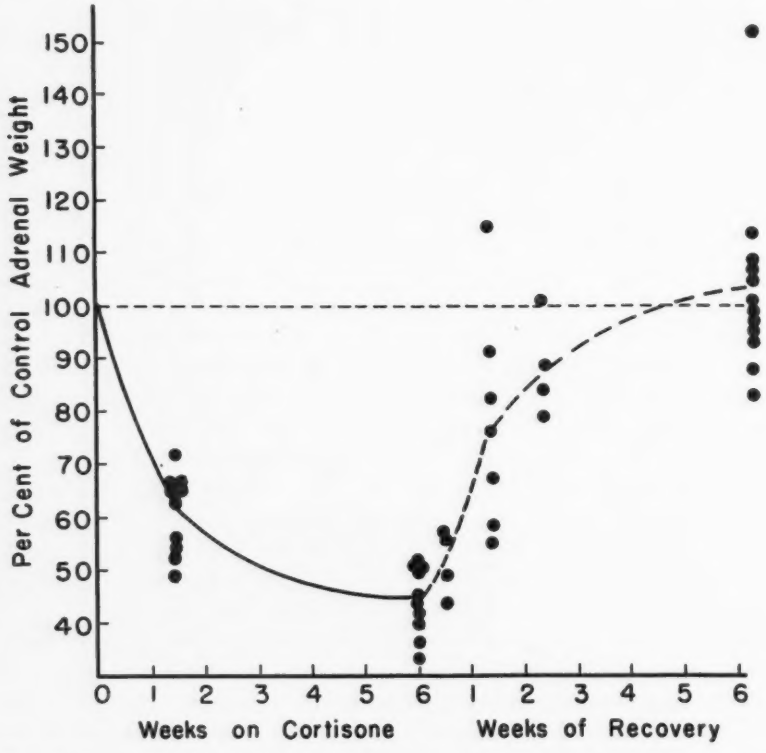

FIG. 3. Course of adrenal atrophy during cortisone treatment in rats, and recovery after discontinuing injections. 100 per cent = mean weight of adrenals of controls of comparable body weight. Dots = individual data. Dose of cortisone: 3 mg. per rat daily.

Table 1 shows the changes in cholesterol and ascorbic acid content of the adrenals during the period of treatment, and in recovery. It is evident that the dose of cortisone used produced a profound fall in concentrations of cholesterol and ascorbic acid, but that recovery from this effect took place quite promptly, and was complete within 10 to 17 days.

The histological changes produced in the adrenal by the cortisone injections were essentially the same in the animals observed after 10 days of

treatment as in those observed after 6 weeks of injections. The cortex was markedly decreased in width, due to reduction in size of the cells of the fascicular and reticular zones. There was virtually complete loss of lipid from these cells. The cells of the subcapsular zone (glomerulosa) were practically unaltered. In this respect, the effect of treatment with cortisone resembled that of hypophysectomy, and was unlike that seen after administration of desoxycorticosterone or prolonged NaCl feeding. In the latter cases, the glomerulosa becomes atrophic (Carnes, Ragan, Ferrebee and O'Neill 1941).

TABLE 1. CHOLESTEROL AND ASCORBIC ACID CONCENTRATIONS IN THE ADRENAL GLANDS OF RATS DURING TREATMENT WITH CORTISONE ACETATE, 3 MG DAILY, AND RECOVERY FROM TREATMENT

	Controls	During treatment		After cessation of treatment		
		10 days	6 weeks	10 days	17 days	45 days
Av. cholesterol mg. per gm. of adrenal tissue	49	23	11.5	42	49	54
Av. ascorbic acid mg. per gm. of adrenal tissue	3.5	3.1	2.4	2.9	3.2	3.2

The reversal of the atrophic changes after discontinuance of treatment was very prompt. Within 4 days, many of the cells of the zone fasciculata and reticularis had regained cytoplasmic lipid, and in many areas, the amount of lipid present was even greater than in the glands of normal rats. After 10 days of recovery, the appearance of the glandular parenchyma was nearly normal. The glands of animals sacrificed 17 days or 45 days after withdrawal of treatment were essentially normal in appearance. Photomicrographs illustrating the changes in the glands during treatment and restitution after cessation of injections are presented in Fig. 4, 5, 6, and 7

Effects on other organs. The administration of cortisone had no effect upon the size of liver, testes, pituitary gland, kidneys, or heart. The thyroid glands of the injected animals had a slightly higher average weight than did controls of similar body weight, but the difference was probably not significant. In 10 control animals weighing from 181 to 212 gm. (average 192 gm.), the mean thyroid weight was 12.8 mg. (range 10.4–16.8), compared to a mean of 15.6 mg. (range 12.2–18.4) in 10 animals (average body weight 192 gm.) injected with cortisone for 6 weeks. Histologically, both the thyroid glands and pituitary glands of the treated animals appeared entirely normal. There were no changes in the basophiles of the hypophyses, such as were described by Crooke (1935) in patients with Cushing's syndrome.

The average combined weight of the seminal vesicles and prostate of the animals treated for 6 weeks was 1.40 gm., compared to 0.49 gm. for con-

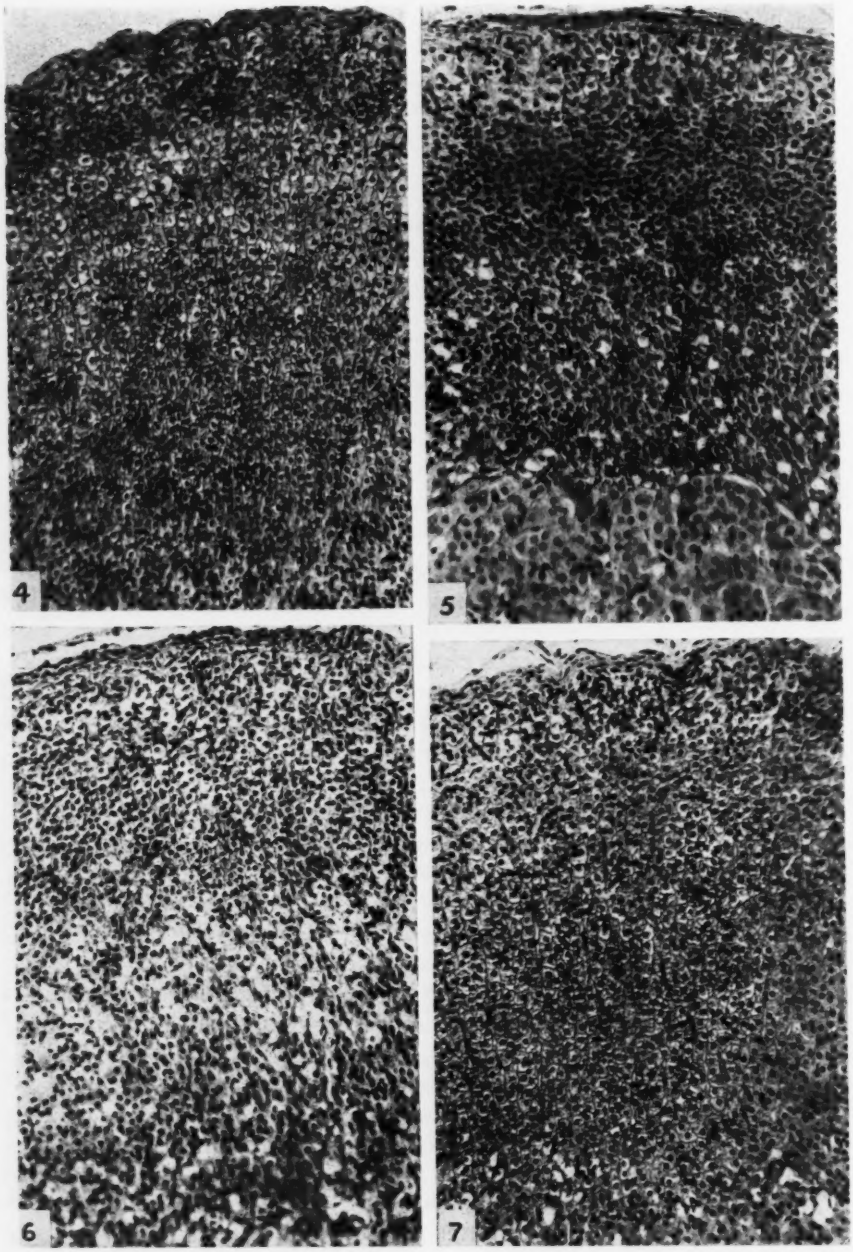

PLATE I. Sections of rat adrenals ×230. Hematoxylin-eosin.

trols of similar body weight. However, this result does not necessarily demonstrate an androgenic action of cortisone, since age and degree of sexual maturity are also factors in the weights of these organs, and these structures were smaller in the treated animals than in controls of similar age. Therefore, these data merely demonstrate that cortisone does not inhibit the growth of the accessory reproductive structures as markedly as it does general body growth. Cortisone does not affect the atrophy of the seminal vesicles and prostate glands which occurs following castration in rats (Winter, unpublished data).

Hausberger (1937) has shown that the fat body attached to the rats testis is a structure of highly reproducible weight. Changes in the weight of this structure closely parallel changes in neutral fat content of the carcass (Schiffer and Wertheimer 1947; Stoerk and Porter 1950). The "testicular fat body" was dissected out and weighed in the present experiments. In the 10 rats sacrificed after 6 weeks on cortisone, the mean weight of this structure was 1.95 gm., compared to 1.30 gm. in 10 controls. Thus the fat body in the treated rats was 50% heavier than in the controls. This effect was reversible, however, as in those sacrificed 45 days after discontinuing the injections, the weight of the fat body was the same as for controls of similar body weight (2.16 gm. compared to 2.33 gm.).

As expected, cortisone treatment led to reduction in size of the spleen and thymus (Wells and Kendall 1940), especially the latter. At the end of 6 weeks of treatment, the mean weight of the spleen was 0.40 gm., and of the thymus 0.036 gm., compared to 0.55 and 0.49, respectively, in controls of similar body weight. Partial reversal of this effect took place rather quickly, as 4 days after cessation of treatment mean spleen and thymus weights for the injected animals were 0.49 gm. and 0.16 gm. respectively. However, even after 45 days of recovery, the thymi had not recovered full size, as they weighed 0.21 gm. on the average, compared to 0.43 gm. for controls of the same body weight. Histologically, the thymus glands of the

FIG. 4. Adrenal of control rat (Group IIIb). Throughout most of the tissue, there is an abundance of foamy cytoplasm, containing lipid.

FIG. 5. Rat of Group I, after receiving 3 mg. of cortisone acetate daily for 6 weeks. The width of the cortex is reduced, and the cells of the reticular and fasicular zones are reduced in size, due to loss of cytoplasmic lipid. Scattered through the reticular zone, there are a number of large vacuolated cells; these cells contain no demonstrable lipid when stained with Sudan IV. The cells of the glomerular zone are essentially normal in appearance.

FIG. 6. Rat of Group I, 4 days after cessation of treatment with cortisone acetate, 3 mg. daily for 6 weeks. Many of the cells of the fasicular and reticular zones have regained their cytoplasmic lipid, apparently even in excess.

FIG. 7. Rat of Group I, 17 days after cessation of treatment with cortisone acetate, 3 mg. daily for 6 weeks. The gland has resumed its normal microscopic appearance.

treated rats appeared to be normal within 10 to 17 days after discontinuing injections.

Sections of ribs and of tibia were examined histologically, since osteoporosis is a regular finding in patients with Cushing's syndrome. There was striking reduction in the width of the epiphyseal cartilage, but no appreciable rarification of cortical or cancellous bone in the treated animals. There were, however, osteoporotic changes in the paired weight controls presumably as a result of inanition. The present findings on bone histology are similar to those of Baker and Ingle (1948) in animals treated with ACTH.

Other histological changes produced by the injections, and worthy of mention, though apparently of minor importance were: the presence of a few scattered cells in the reticular zone of the adrenal cortex showing "hydropic" degeneration, deposits of basophilic material of unknown nature in the Kupffer cells, and the appearance within the Leydig cells in the testes of coarse granules with metachromatic staining properties. A detailed account of the histological changes observed in the treated animals will be published elsewhere.

Effect on blood constituents. Table 2 presents the principal findings on the biochemical constituents of the blood. The injections had no effect on total serum protein, plasma fibrinogen, glucose, urea, allantoin, calcium, sodium, or potassium. There was a decrease in α-globulin, an increase in γ-globulin, with only minor changes in A/G ratio. The changes in both the α-globulin and γ-globulin fractions proved to be reversible within 17 days after withdrawal of the drug. Changes in β-globulin were irregular.

TABLE 2. BIOCHEMICAL DATA ON RATS DURING TREATMENT WITH CORTISONE ACETATE, 3 MG. DAILY, AND RECOVERY FROM TREATMENT

Constituent of serum or plasma	Controls	During treatment		After cessation of treatment		
		10 days	6 weeks	10 days	17 days	45 days
Serum protein, gm. per cent	5.1	4.9	5.0	5.1	5.1	5.9
Serum albumin, gm. per cent	1.67	1.65	1.87	2.00	2.02	1.92
α-globulin, gm. per cent	2.19	1.70	1.57	1.51	2.06	2.57
β-globulin, gm. per cent	0.76	0.60	0.73	0.98	0.80	1.14
γ-globulin, gm. per cent	0.49	0.90	0.79	0.57	0.26	0.31
A/G ratio	0.50	0.53	0.61	0.66	0.65	0.48
Fibrinogen, mg. per cent	227	—	220	250	230	—
Cholesterol, total, mg. per cent	72	110	122	85	87	103
Cholesterol, ester, mg. per cent	61	95	111	74	72	94
Cholesterol, ester: total ratio	0.84	0.86	0.92	0.87	0.83	0.91
Glucose, mg. per cent	148	140	155	155	165	150
Urea, N, mg. per cent	20	20	20	17	21	20
Allantoin, mg. per cent	3.0	3.7	2.4	2.8	2.9	2.6
Calcium, mg. per cent	10.1	9.0	10.0	10.5	11.5	10.8
Sodium, mg. per cent	338	358	337	316	316	—
Potassium, mg. per cent	18	18	20.5	18	17.5	—
Alkaline phosphatase, units	23	25	38	23	26	18

Serum cholesterol increased about 70% with no significant change in ester/total ratio, and alkaline phosphatase rose about 65% during the course of injections; both returned to normal levels upon cessation of treatment.

In addition to these studies on blood, determinations were also made of fat, nitrogen, phosphorus, and glycogen in the liver and of nitrogen phosphorus, and glycogen in the kidney. In all cases, values within normal limits were found. In this connection, it is to be emphasized that the animals were fed *ad libitum*, and in those animals sacrificed after 10 days or 6 weeks of treatment, 24 hours had elapsed since their last dose of cortisone. Thus, the acute effects of cortisone on fasted animals were avoided. Our findings do not deny the reports of others (Long, Katzin and Fry 1940; Long, 1942; Reinecke and Kendall 1943; Pabst, Sheppard and Kuizenga 1947) on the acute effects of cortisone on fasted animals, but demonstrate the absence of chronic effects in fed animals on such factors as liver glycogen, which are notably labile in acute experiments.

DISCUSSION

The prolonged administration of large quantities of cortisone might have been expected to cause changes related to toxicity of the steroid. All significant alterations observed in this study, however, appear to be a consequence of excessive hormonal activity, rather than of toxicity of cortisone in the usual toxicological sense. In spite of the many dissimilarities between the murine and the human organism, several features of human hyperadrenocortinism (Cushing's syndrome) have been reproduced in rats in the present experiments.

The signs of overdosage with cortisone observed in the present experiments were of a temporary nature, and discontinuance of the hormone was followed by prompt return toward a normal state. The reversibility of the changes in the rat is the counterpart of the regression of symptoms in cases of Cushing's syndrome when a causative adrenal tumor is removed, and of the disappearance of changes resembling Cushing's syndrome when cortisone is withdrawn from patients showing such changes under cortisone therapy.

The relatively large food consumption with retarded body growth observed in the rats during treatment with cortisone resembles that seen in hypopituitarism, which can be reversed by somatotrophic hormone (STH). In the present experiments, no changes in weight or morphology of the pituitary were seen, but suppression of the release of ACTH is a generally accepted action of cortisone. The effects of cortisone on body growth could be due either to suppression of the release of STH, or to

antagonism to the peripheral action of STH. Further experimentation may decide this point.

The suppression by cortisone of the release of ACTH from the pituitary probably explains the adrenal atrophy produced by cortisone; therefore, the reversal of the atrophy after discontinuance of cortisone would be expected, since cessation of injections would permit ACTH to be released. It is clear that even the large doses of cortisone administered in these experiments produced no permanent damage to the adrenal gland. Definite histological evidence of restoration of the adrenal cortex was seen in animals sacrificed four days after cessation of treatment.

The relative increase in depot fat observed in these experiments is probably analogous to the peculiar obesity observed in hyperadrenocortinism in man (Cushing's syndrome). Kochakian (1944) and Kendall (1945) described an increase in carcass fat in mice after administration of 11-dehydrocorticosterone. The effect is probably related to the observation of Stoerk and Porter (1950) that cortisone prevents the loss of fat in animals during food restriction. The reversal of this effect after injections are discontinued is comparable to the disappearance of the adiposity in cases of Cushing's syndrome when a causative adrenal tumor is removed (Walters, Wilder and Kepler 1934). The increase in plasma cholesterol is also probably related to a disturbance in fat metabolism. It has been seen in man (Ragan, Grokoest and Boots 1949, Adlersberg, Schaefer and Dritch in press).

The effect on hair growth is presumably due to the production of atrophic changes in accessory structures of the skin, such as are known to occur in Cushing's disease, and have been described after local application of cortisone (Whitaker and Baker 1948, 1949) and injection of ACTH (Baker, Ingle, Li and Evans 1948). It is not known whether this effect is a consequence of the nitrogen loss produced by cortisone, or is some more specific local effect upon the skin.

The significance of the changes in α- and γ-globulins is not clear: The increase in γ-globulin appears to be confirmatory to White and Dougherty (1945). However, Li and Reinhardt (1947) failed to demonstrate any effect of ACTH upon γ-globulin, and Eisen *et al.* (1947) also obtained negative results with Upjohn's lipo extract. The determinations of the serum globulin fractions in the present experiments were made by a salt fractionation procedure, and the findings need to be confirmed by electrophoresis and ultracentrifugation. The changes found proved to be reversible upon discontinuance of injections.

The increase in alkaline phosphatase in the absence of bone changes and without morphological or functional (cholesterol ester: total cholesterol ratio; serum albumin: globulin ratio) evidence of liver damage,

suggest a direct effect upon either production or activation of the enzyme.

Although all the changes observed in this study were reversible upon discontinuance of injections, it is possible that if injections had been continued for a longer period of time irreversible changes may have been produced.

SUMMARY

Cortisone acetate was administered subcutaneously to young adult male rats, 3 mg. daily for 6 weeks. Growth ceased during treatment. The cortisone-treated rats consumed 47% more food than a control group with food intake restricted to maintain the same body weight as the injected animals. The injections completely suppressed regrowth of hair in an area denuded by plucking. Six weeks of treatment produced over 50% atrophy of the adrenal glands. The atrophy involved the zonae fasciculari and reticularis, but the zona glomerulosa was practically unaltered. The cholesterol and ascorbic acid concentrations in the adrenal glands were markedly reduced.

The thymus gland became extremely atrophic during the course of treatment, and the spleen was moderately reduced in size. There was an increase in depot fat. Other organs studied were virtually unaffected by the hormone. Serum α-globulin was decreased, and γ-globulin (salt fractionation procedure) was increased during treatment. Serum cholesterol and alkaline phosphatase were also increased. Other plasma and serum constituents were unchanged. All these changes proved to be reversible, generally within 10 to 17 days after discontinuance of the injections. No chronic effects were found upon liver fat, nitrogen, phosphorus, or glycogen, or upon kidney nitrogen, phosphorus, or glycogen.

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NOTES AND COMMENTS

ANTAGONISM OF ESTROGENS BY TESTOSTERONE PROPIONATE IN HYPOPHYSECTOMIZED FEMALE RATS

Crude androgenic extracts of testis inhibit the estrous cycle of the intact rat, but this effect can readily be counteracted by concurrent administration of a urinary gonadotropin preparation. It was concluded (Ihrke and D'Amour, 1931; Moore and Price, 1932) that androgens exert their effect on estrus by depressing the secretion of gonadotropin by the pituitary, thus curtailing the normal stimulus for production of estrogens by the ovaries. Subsequently, however, it was found that after ovariectomy of mice and rats, pure androgens (testosterone and its propionate) were still capable of inhibiting the vaginal estrus produced in these animals by administration of exogenous estrogens (Robson, 1936; Hain 1937). It is therefore clear that the depressant effect of androgens on vaginal estrus is at least not entirely dependent on the pituitary-ovary pathway.

The following experiments on hypophysectomized rats demonstrate that, independent of its effect on the pituitary, testosterone propionate can inhibit the estrous response of the rat vagina to estrogens, whether of endogenous or exogenous origin.

EXPERIMENTAL

Two groups of Sprague-Dawley female rats were used. All rats in each group were of the same age, and at the time of operation their body weights fell within a 20-gram range.

TABLE 1. INHIBITION BY TESTOSTERONE PROPIONATE OF VAGINAL ESTRUS INDUCED IN HYPOPHYSECTOMIZED RATS

Treatment (total dose)	No. of rats	Per cent in estrus
Groups A: Rats Hypophysectomized		
0.4 mg. gonadotropin plus oil	22	59
0.2 mg. gonadotropin plus oil	22	41
0.4 mg. gonadotropin plus 0.25 mg. testosterone propionate	22	0
0.2 mg. gonadotropin plus 0.25 mg. testosterone propionate	22	9
Group B: Rats Hypophysectomized and Spayed		
1.5 gamma estrone plus oil	18	89
1.5 gamma estrone plus 1.0 mg. testosterone propionate	18	0
1.5 gamma estrone plus 0.25 mg. testosterone propionate	18	0

The rats in Group A were hypophysectomized over a 5-day period at 57–61 days of age. During the subsequent weeks varying dosages of a sheep pituitary preparation were administered to determine the optimum dosage level and proper time intervals at which to sample the vaginal contents. Eight weeks after hypophysectomy the gonadotropin preparation in saline and testosterone propionate in oil (or oil alone) were both administered subcutaneously twice daily for four days. The injections were made at different sites. Vaginal smears were examined 78 and 90 hours after the first injection, and only a fully cornified smear was classified as positive.

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The rats in Group B were spayed at 58 days of age and hypophysectomized at 70 days. 72 days later (after several preliminary experiments with estrogen dosage) all the rats were given a "priming dose" of 0.5 gamma of estrone in oil, and 48 hours later a second injection of 1.0 gamma of estrone, following the estrogen assay procedure of Koch (1942). Testosterone propionate in oil (or oil alone) was administered at the same time as the second dose of estrone, but at a different site. All injections were subcutaneous. Vaginal smears were examined 48 and 52 hours after the last injection.

The results are shown in Table 1.

DISCUSSION

That the testis elaborates a hormone capable of inhibiting the pituitary has been inferred from the observation that castration is followed by increased secretion of pituitary gonadotropin. Vaginal estrus is one of the phenomena which is dependent on the pituitary, and since adequate doses of testosterone as well as crude extracts of testis will inhibit estrus, it would appear reasonable that testosterone might be responsible for pituitary inhibition by the testis.

However, our own experiments and those of others have shown that the estrus-inhibiting action of testosterone is not abolished by ovariectomy and hypophysectomy. Without questioning the suppressive action of adequate concentrations of testosterone on gonadotropin secretion by the pituitary, it therefore seems likely that inhibition of estrus by testosterone in the intact rat may be partially or entirely a local effect on the vagina. Furthermore, Koch (1948) found that the estrogen-antagonizing activity of testis extracts could be concentrated in a phospholipin fraction devoid of androgenic activity. It was also noted that the estrus-inhibitory property of testosterone may be of pharmacological rather than of physiological significance, and in our opinion the question, suggested by Koch's observations, of the existence of an additional testis hormone capable of inhibiting the pituitary deserves further investigation.

SUMMARY AND CONCLUSION

Concurrent administration of testosterone propionate prevents the appearance of vaginal estrus otherwise readily obtainable (a) by administration of pituitary gonadotropin to hypophysectomized rats with intact ovaries and (b) by administration of estrone to hypophysectomized spayed rats. On the basis of these experiments and other evidence cited it is concluded that the inhibitory action of testosterone on the estrous cycle of the rat may be independent of the pituitary and the ovary.

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